An Extracellular Matrix-localized Metalloproteinase with an Exceptional QEXXH Metal Binding Site Prefers Copper for Catalytic Activity

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The extracellular matrix (ECM) of the simple multicellular organism Volvox contains many region-specific morphological elements and mediates a variety of developmental and physiological responses by modification of its components. The fact that >95% of the mature organism is ECM makes Volvox suitable as a model system for ECM investigations. VMPs are a family of Volvox genes that are homologous to zinc-dependent matrix metalloproteinases (MMPs). Here we describe the identification and purification of the first VMP protein, VMP3. The 470-kDa VMP3 glycoprotein is localized within the ECM, and its biosynthesis is induced by the sex pheromone. The metal binding motif of VMP3 is QEXXH, not HEXXH as known for ~1300 other metalloproteinases. VMP3 shows proteinase activity and is inhibited by EDTA or the MMP inhibitor GM 6001, but in contrast to all known proteinases, VMP3 clearly prefers copper for activity rather than zinc. The exchange from Q to H within the QEXXH motif abolishes its copper preference. The unique properties of VMP3 suggest a novel type of metalloproteinase.

The well timed breakdown of an ECM is indispensable for embryonic development, morphogenesis, reproduction, tissue resorption, or remodeling. In vertebrates, one of the major groups of enzymes that degrade ECM components is the matrix metalloproteinase (MMP) family. MMPs regulate many biological processes and are themselves closely regulated. The effectors that induce MMP synthesis not only include growth factors and cytokines, but also physical stress and cell-matrix cell-cell interactions. The MMPs are among the hydrolyases in which the nucleophilic attack on a peptide bond is mediated by a water molecule, a characteristic shared with the aspartic peptidases. Although in the metallopeptidases the divergent metal cation zinc activates the water molecule (1), the zinc is held in place by three amino acid ligands, and consequently, the catalytic domain of MMPs contains a conserved zinc binding motif, which is HEXXHXXGXXH.

The occurrence of MMPs is not restricted to vertebrates, lower animals like sea urchins or Caenorhabditis have also been shown to possess MMP homologues, as well as lower plants like Chlamydomonas and higher plants, like Arabidopsis, soybean, or cucumber. Recently, four MMP-related genes, named VMP genes, were identified in the multicellular green alga Volvox carteri (2). Like MMPs, the VMP genes code for proteins with a putative proteinase domain and a proline-rich, probably rod-like domain (HR domain). However, there is one hitch with the presumptive zinc binding site of all VMPs: it is QEXXHXXGXXH not HEXXHXXGXXH.

Despite the general simplicity of Volvox, which has only two cell types, somatic and reproductive, its ECM is surprisingly complex (3). It consists of many region-specific, anatomically distinct structures arranged in a defined spatial pattern. These structures are modified under physiological, metabolic, or developmental control. The main zones of the ECM are named FZ (flagellar zone), BZ (boundary zone), CZ (cellular zone), and DZ (deep zone) (3). The BZ includes the components of the ECM that, except in periflagellar regions, appear to be continuous over the surface of the organism. The CZ includes components lying internal to BZ and exhibits specializations around individual cells. The DZ contains all ECM components internal to the CZ, fills the deepest regions of the spheroid, and is far the largest region.

One important event in development of Volvox is the change from vegetative to sexual development. The stimulus for switching from the vegetative to the sexual mode of reproduction in V. carteri is known to be a sex-inducing pheromone (4, 5). This 32-kDa glycoprotein is one of the most potent biological effector molecules known, since it still works at concentrations as low as 10⁻¹⁶ m. The first responses to the pheromone are structural modifications within the ECM (6) as well as changes within the mRNA population. By differential screenings of cDNA libraries (vegetative versus sexually induced) several new genes have been discovered; four of these genes are VMPs (2).

In this paper we describe the identification and purification of VMP3 protein, the first ECM-localized metalloproteinase from Volvox. Among other unusual properties, the enzyme prefers copper for activity rather than zinc, suggesting a new type of metalloproteinase. Transgenic algae carrying point mutations within the QEXXH motif were generated to give detailed information about the metal binding site.

EXPERIMENTAL PROCEDURES

Culture Conditions—The female V. carteri f. nagariensis strains HK10 (wild type) and 153–48 (nitA⁻) were obtained from R. C. Starr (University of Texas, Austin, TX) or D. L. Kirk (Washington University, St. Louis, MO). Cultures were grown in Volvox medium (7) at 28 °C in a 16 h dark/16 h light (10,000 lux) cycle (4).

Separation of Somatic Cells, Reproductive Cells, and DZ—Spheroids were broken up by forcing them through a 0.4-mm hypodermic needle,
followed by centrifugation at 20,000 \( \times g \) for 5 min. The clear, highly viscous supernatant is the so called DZ extract. Other components were fractionated as described (2).

**Heterologous Expression for Antibody Production—cDNA encoding amino acids 22–475 of VMP3 was amplified by polymerase chain reaction (PCR) (BL21(DE3)).** Expressed in New Delhi niR1 and VMP3H sites were engineered along with a (His),-tag sequence; the sense primer was 5'-ACATATGGCTCCA-

**Antibodies and Western Blotting**—Antibodies were raised in rabbits and purified on protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). Detection of electroblotted proteins was performed by using the VMP3 antibody at a 1:25,000 dilution and an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) at a 1:10,000 dilution. Staining with nitroblue tetrazolium was performed on the same template with the sense primers 5'-CAATGGACGACCAGCATG (anti-VMP3) and 5'-CTGACGATGCATGTCG (VMP3-H), 5'-AGCATCATG-

**Immunoprecipitation**—100 \( \mu l \) of magnetic beads (Dynabeads 450, Dynal, Oslo, Norway) conjugated with sheep anti-rabbit-IgG antibodies were washed with phosphate-buffered saline-Tween, mixed with VMP3 rabbit immune serum (or preimmune serum) at a ratio of 1:20 (v/v), and incubated on a rotary shaker for 15 h at 4 °C. After four washing steps, extracts containing VMP3 were added to the beads-bound antibodies. Incubation and washing followed. Finally, the antigen was removed from the beads by adding standard gel loading buffer without dithiothreitol (prior to in-gel assays).

**In-gel Proteinase Activity Assay—Samples** were mixed with gel loading buffer without thiol reagents and loaded (without heating) onto SDS-polyacrylamide gels (8\% w/v), containing 7.5 mM SDS, 3% \( N_7 N_7 N_7 -\)tetramethylethylenediamine, and 0.3 mg/ml ammonium persulphate (8). Electrophoresis was in 66 mM sodium phosphate buffer. H 7.0, 2.5% SDS at 12 mA and 22 °C for 24 h.

**Deglycosylation—VMP3 was dissolved in anhydrous hydrogen fluoride and incubated at 0 °C for 90 min (9).**

**In-gel Proteinase Activity Assay—Samples** were mixed with gel loading buffer without thiol reagents and loaded (without heating) onto SDS-polyacrylamide gels (8\% w/v) separation gel, 5% stacking gel) supplemented with 0.2% gelatin. After electrophoresis at 14 mA for 3 h, in-gel proteinase activity was performed in Volvox medium supplemented with 0.02% NaN\(_2\), 0.1% ZnCl\(_2\), and 1% Triton X-100 for 48 h at 28 °C, followed by incubation in the same solution, but without Triton for 24 h (10). Gels were stained with Coomassie Blue for 30 min and then destained. Potential inhibitors were added in concentrations that are customary in proteinase assays, i.e. 1 mM EDTA, 1 mM EGTA, 2 mM 1,10-phenanthroline, 0.5 mM phenylmethylsulfonyl fluoride, 10 \( \mu M \) GM 6001, or 10 \( \mu M \) dithiothreitol.

For assaying metal-ion specificity in the experiments described in the results section, renaturation was in the presence of 1 mM EDTA plus 2 mM of the metal-ions Zn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), or Fe\(^{2+}\). In addition, studies on Zn\(^{2+}\) and Cu\(^{2+}\) replacement were carried out at a series of concentrations from 10 \( \mu M \) to 2 mM, showing that the described effects are also true for lower concentrations (down to 10 \( \mu M \)); no inhibitory effects were detectable within the range of concentrations used.

**Gel Documentation and Quantification—Gel documentation and quantification was by using a Photo-print system (Vilber Lourmat, Marne-la-Vallée Cedex, France) and the Bio-1D Software (version 5.01, Vilber Lourmat, France).**

**Construction of Gene Derivatives—A PstI fragment coding for the complete HR domain of VMP3 was used to generate the modifications in VMP3a, VMP3b, and VMP3y.** There are two EcoRI sites within the PstI fragment. The 423-bp EcoRI fragment was removed and the ends ligated in-frame, resulting in the deletion within VMP3y. In addition, there is an AatII site in the middle of the PstI fragment. By using recombinant PCR (11) an artificial AatII site was added either at the 5' or 3'-end of the HR domain, which resulted in shortened PstI-AatII (VMP3a) or AatII-PstI (VMP3b) fragments, respectively. The primers that introduced the artificial AatII site (underlined) were 5'-TACGAC-

**RESULTS**

**In Search of VMP3 Protein—VMP3 cDNA encoding the putative proteinase domain was cloned into E. coli expression vectors and the heterologously expressed VMP3 proteins were found in inclusion bodies, even if an E. coli signal peptide for periplasmic localization was added. Heterologously expressed VMP3 proteins were purified and refolding was done in the presence of metal ions and detergents following published methods (16–18). Finally heterologously expressed VMP3s were analyzed for proteinase activity. Neither in solution nor in in-gel activity assays (10) a proteinase activity could be detected (data not shown). In a different approach, the VMP3 variant carrying the E. coli signal peptide for periplasmic localization was co-expressed with the protein disulfide isomerase to reduce the likelihood of problems with disulfide bonds; refolding also was done in the presence of glutathione as described (19). These additional experiments did not either lead to a measurable proteolytical activity (data not shown). However, heterologously expressed ECM enzymes often do not show activity due to the lack of essential posttranslational modifications. Therefore, we intended to search for the VMP3 polypeptide in wild-type Volvox. For that purpose the heterologously expressed proteinase domain of VMP3 was used for generation of polyclonal antibodies. In Western blots the antibody detected a single band in Volvox lysates (Fig. 1A), indicating the existence of a high molecular mass VMP3 protein.**

**VMP3 Is Localized within the ECM Zone DZ—After mechanical disruption of a Volvox alga, its components can be separated from each other by filtration and centrifugation steps. All fractions were analyzed separately by SDS-PAGE followed by Western blotting using the VMP3 antibody. As documented in Fig. 1B, VMP3 is localized only within the DZ, thus being a component of the ECM that fills out the extracellular interior of the spheroid.**

In a different approach VMP3 was localized by fine structure immunocytology using the VMP3 antibody. Immunogold-labeled and heavy metal counterstained specimens were examined by transmission electron microscopy as described (20) (data not shown). Gold particles marking the localization of
VMP3 were uniformly distributed within the DZ (DZ1 and DZ2), confirming the results obtained with the above biochemical approach.

Synthesis of VMP3 Is Stimulated by the Sex-inducing Pheromone—The kinetics of accumulation of VMP3 mRNA in response to the pheromone was analyzed in detail by RT-PCRs. There is a low level of VMP3 mRNA detectable throughout the life cycle even in vegetatively grown algae, but the VMP3 mRNA level increases abruptly after pheromone addition (Fig. 1C). Subsequent to sexual induction, RT-PCRs give a strong signal of constant intensity for at least 18 h, whereas VMP3 protein levels exhibited a steady-going increase after pheromone addition (Fig. 1D).

Purification of VMP3 from Wild-type Volvox—To allow a detailed characterization of VMP3 and to prove identity with the immunoreactive material, VMP3 was purified from wild-type Volvox. For this purpose a DZ extract was fractionated by anion exchange chromatography at pH 9.0 on a QAE-Sephadex column. Elution of VMP3 was at 100 mM NaCl–. Final preparative SDS-PAGE (lane 5). The samples loaded onto the gels in A and B are identical, but detection differs: A, Western blot using the VMP3 antibody. B, silver stain. C, N-terminal sequences of VMP3 deduced from cDNA or determined by Edman degradation of purified, deglycosylated VMP3. Leader peptide cleavage sites are indicated. Hyp = hydroxyproline.

Another striking feature was revealed by N-terminal sequencing: even the very first proline of the mature VMP3 turned out to be modified to hydroxyproline. In the ECM proteins found so far, hydroxyprolines are strictly confined to the HR domains, which consist almost exclusively of hydroxyprolines, whereas other domains are completely devoid of hydroxyprolines (21). N-terminal sequencing does not run into the HR domain of VMP3 but into the proteinase domain, which is not proline-rich.

Finally, N-terminal sequencing shows that only the signal peptide was cleaved off in mature VMP3, but no additional propeptide, which has to be cut off in most MMPs for enzyme activation (1).

Calculated and In-gel Determined Sizes of VMP3 Differ Extremely—A molecular mass of 70 kDa is calculated for the mature VMP3, but on 6% SDS-polyacrylamide gels the protein hardly enters the gel. To give a relatively precise molecular mass, non-standard SDS-PAGEs for high molecular mass proteins were used (8). On these gels VMP3 exhibits ~470 kDa (Fig. 3A), and thus the molecular mass on the gel is almost 7-fold higher than expected. The extracellular localization of VMP3 and the problems in N-terminal sequencing without preceding hydrogen fluoride treatment suggest an extensive glycosylation of VMP3. Actually, deglycosylation reduces the mass of VMP3 to about a fourth, that is ~125 kDa (Fig. 3B).

Nevertheless, the apparent size of the deglycosylated VMP3 is still significantly larger than calculated. The extreme (hydroxy)proline content (~25%) explains the difference between observed and calculated molecular masses, since stretches of poly(hydroxy)proline possess a reduced ability to bind SDS (22).

VMP3 Demonstrates Proteinase Activity—Purified VMP3 from wild-type Volvox was analyzed for proteinase activity by using an in-gel assay (10). For that purpose SDS-polyacrylamide gels were cast in the presence of 0.2% gelatin, and electrophoresis was carried out in the absence of thiol reagents.
After renaturation and incubation, negative staining was achieved with Coomassie Blue. In this assay a proteinase activity was detectable, i.e. gelatin was accepted as an artificial substrate (Fig. 3C). In contrast to gelatin, casein, which is otherwise often used in activity assays, was not an acceptable substrate (data not shown). To prove the identity of the purified protein and activity, the same sample was investigated by a standard SDS-polyacrylamide gel stained with silver, a Western blot, as well as an activity gel (Fig. 3C). In addition, an immunoprecipitation experiment was carried out, in which extracts containing the VMP3 antigen were mixed with the VMP3 antibody, and the precipitate was subsequently analyzed by the activity assay. A VMP3 activity band was detected in the precipitate, whereas control experiments with preimmune serum did not produce any signal (Fig. 3D).

To get information about the real substrate of VMP3, radioactive in vivo pulse labeling experiments were carried out, and purified, radioactive DZ preparations (23) were used as a substrate. In addition, there is a way to get otherwise insoluble ECM components into a soluble form using Ellman’s reagent (24). All these soluble or solubilized ECM components were used as potential substrates in proteinase assays, which were analyzed by fluorography, but no significant digestion of DZ or other ECM components was detectable (data not shown). However, we know that there are VMPs as well as proteinases other than VMPs in the DZ, which might have cleaved the real substrate of VMP3 already in vivo or during preparation of these extracts. Consequently, it is unclear whether the ECM preparations still contained a significant amount of cleavable substrate when we did the activity assays.

A Bulky VMP3 Proteinase Made Handy—The high molecular mass and glycosylation kept us from characterizing VMP3 in more detail. This problem was solved by homologous expression of VMP3 variants with a clearly reduced molecular mass. Since the HR domain seemed to be only a structural component, we reduced its extent. The derivatives VMP3<sub>e</sub>, VMP3<sub>β</sub>, and VMP3<sub>γ</sub> carry deletions at the amino end, the carboxyl end, or in the middle of the HR domain, respectively (Fig. 4A). Volvox algae were transformed with the corresponding gene constructs using a particle gun, and transformants were obtained for each of the three variants. Western blots of wild-type and transgenic strains expressing VMP3<sub>α</sub>, VMP3<sub>β</sub>, or VMP3<sub>γ</sub>, respectively, are shown in Fig. 4B. The VMP3<sub>α</sub> antibody, which is directed against the proteinase domain, was used for detection. The apparent molecular mass is gradually reduced from ~470 kDa in VMP3<sub>α</sub> to ~190 kDa in VMP3<sub>β</sub>, ~130 kDa in VMP3<sub>β</sub>, or ~120 kDa in VMP3<sub>γ</sub>. Fortunately, the extensive deletion within the HR domain had no effect on proteinase activity, and all variants are easily distinguished from wild-type VMP3<sub>α</sub> in the in-gel assay (Fig. 4C). The smallest variant, VMP3<sub>γ</sub>, was used for all further investigations.

VMP3 Enzyme Is Inhibited Just as MMPs—For a detailed characterization of VMP3<sub>γ</sub> quantification of enzyme activity was necessary. At present, activity assays are only feasible within a gel, thus quantification of VMP3<sub>γ</sub> enzyme activity also had to be carried out within the gel. Therefore, increasing amounts of DZ extract of the VMP3<sub>γ</sub>-expressing transformant were analyzed by the in-gel assay (Fig. 5A), and activity bands were quantified using a gel documentation and quantification system. Fig. 5B shows that there is a range of linear correlation between the amount of VMP3<sub>γ</sub> loaded onto the gel and the determined relative activity, thereby allowing us to quantify activity within the gel. By means of this quantification method, identical amounts of VMP3<sub>γ</sub> were assayed in the presence of different potential inhibitors. Fig. 5, C and D, show that the metal chelators EDTA, EGTA, or 1,10-phenanthroline as well as dithiothreitol inhibit proteinase activity almost quantitatively, whereas phenylmethanesulfonyl fluoride, which inhibits serine proteinases but not metalloproteinases, has only a minor effect on activity. Aside from metal chelators and dithiothreitol, the MMP inhibitor GM 6001 reduces activity of VMP3<sub>γ</sub> to zero.

Copper, Rather than Zinc, Is Preferred for Proteolytic Activity of VMP3—Sensitivity to metal chelators demonstrated involvement of a metal ion in VMP3<sub>γ</sub> activity. To find out the metal ion specificities, in-gel renaturation was performed in the presence
The Copper Preference of VMP3 Is Almost Abolished by the Q-to-H Exchange—As described above, VMP3γ, carrying the wild-type QEXXH motif, shows a 15-fold higher activity if copper was used instead of zinc. When the same experiment was performed with VMP3γ-H, which has the mutant HEXXXH motif, activity with copper was only 2.5-fold above that for zinc. Thus, the Q-to-H exchange nearly abolished the copper preference.

**DISCUSSION**

**VMP3, a Metal Ion and Its Binding Site**—Sequence comparisons imply that VMPs are members of the MA clan of zinco-dependent metalloproteinases as defined in the MEROPS protease data base (25), although their putative metal binding site is QEXXH instead of HXXXH (Fig. 6A). We speculated that the different amino acid at position 1 of the highly conserved zinc binding motif could be responsible for the preference of VMP3 for copper. Therefore, the QEXXH motif was subjected to mutation analysis, and transgenic *Volvox* strains expressing different VMP3γ variants were generated.

**FIG. 6. Consensus sequence of zinc metalloproteinases and VMPs and localization of point mutations in VMP3γ sequence and their effects on activity.** A, conserved sequences in metalloproteinases and VMPs: MMP-1 = matrix metalloproteinase 1 (α1 collagenase-1) from *Homo sapiens* (33), MMP-2 = matrix metalloproteinase 2 (gelatinase A) from *H. sapiens* (34), At1-MMP = matrix metalloproteinase from Arabidopsis thaliana (16), SMEP1 = metalloendoproteinase 1 from Glycine max (35), Ca1-MMP = matrix metalloproteinase from Cucumis sativus (17). B, analysis of VMP3γ point mutations: equal amounts of VMP3γ (from DZ extract) and of the VMP3γ variants carrying point mutations (VMP3γ-H, VMP3γ-L, VMP3γ-A) were analyzed by Western blot using the VMP3 antibody. C, in-gel activity assay (in the presence of Zn2+) using the same samples as in B. D, the intensities of the bands shown in C were quantified. The result with VMP3γ-H was set to 100%.

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Timing of VMP3 Synthesis, a Hint to Its Biological Function—VMP3 synthesis increases significantly ~3 h after addition of the sex-inducing pheromone, but this is far not the only pheromone-induced protein detectable: beside VMPs, several other ECM glycoproteins have been identified that are synthesized shortly after this stimulus (21, 31). The majority of those proteins fit into a single family of ECM glycoproteins, the pherophorins. Pherophorins represent the earliest biochemical response to the pheromone and they carry a C-terminal domain that shares homology with the sex-inducing pheromone. Interestingly, in some pherophorins this C-terminal domain soon becomes proteolytically liberated (32), and the start of this cleavage process coincides with the onset of VMP3 synthesis. Seen from this angle, members of the VMP family look like candidates for this task, especially since cleavage of pherophorins has been proposed as part of a novel signal amplification process that is required to get the exquisite sensitivity of the sexual inducing system (32). The main objective of future investigations should be the identification of the real substrates of VMPs. Since the ECM shows a distinct structural architecture, and ECM glycoproteins like pherophorins are found in defined ECM zones, each VMP could be responsible for cleavage of a specific ECM glycoprotein, e.g. a pherophorin, within a specific ECM zone. Consequently, VMP3 would be responsible for the DZ.

As demonstrated previously, synthesis of VMP mRNAs is triggered not only by the sex-inducing pheromone, but also by wounding of the organism (2). Thus, the VMP metalloproteinases also seem to serve a function in ECM repair or remodeling after wounding. Apparently, the simple multicellular alga Volvox responds to environmental stimuli and wounding in much the same way as observed in higher organisms.

Interchangeable Modules in ECM Glycoproteins—Strikingly, VMP3, as well as the rest of the VMP family, exhibit not only functional, but also structural, similarities to the MMPs of animals: they all show a modular assembly. In comparison of VMPs with, for example, human MMP-1 (collagenase 1), the following consistencies become apparent: both have a cleavable N-terminal signal peptide, followed by a globular domain carrying the catalytic center. Behind the catalytic domain a proline-rich linker region is attached in both, MMP-1 and all VMPs. In MMP-1 the proline-rich linker region on its part is connected to a hemopexin domain, which may help to bind the enzyme to the ECM (1); similarly, in VMP4 the proline-rich linker is connected to the C1/C2 domains, which seem to be tandemly repeated protein binding sites (2). There are no C1/C2 domains in the VMPs 1–3, but also extended polyhydroxyproline domains are suggested to be responsible for anchoring extracellular proteins within the ECM (21). In contrast to most MMPs, VMP3, and presumably the rest of the VMP family, do not make use of propedeptides for delayed enzymatic activation.

Apart from the HR and proteinase domains of VMPs many other modules with a variety of functions have already been identified in ECM proteins of Volvox (21), suggesting that different modules have been combined or exchanged during evolution to yield chimeric and multifunctional polypeptides.

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