The presence and possible functions of the matrix metalloproteinase collagenase activator protein in developing enamel matrix

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The developing enamel matrix contains mostly amelogenins, which are hydrophobic proline-rich proteins. During amelogenesis, the amelogenins are presumably hydrolysed and removed from the enamel. Recently a number of metalloproteinases that may be important in amelogenesis have been identified in zymograms of the developing enamel matrix. In the present study an antibody specific for the matrix metalloproteinase collagenase activator protein (CAP) was characterized and used to identify this metalloproteinase in enamel. Immunoblotting showed that the CAP proteinase was present in the enamel matrix. Immunohistochemistry confirmed that the proteinase is localized in the enamel matrix, most specifically along the dentino-enamel junction. Purified CAP was found to hydrolyse amelogenin protein. Possible functions of the proteinase in the enamel matrix are discussed.

INTRODUCTION

Tooth enamel is secreted as a protein matrix, composed mostly of amelogenins, which are hydrophobic proline-rich proteins. A gradual loss of amelogenin protein occurs during the secretory stage of enamel development. During the maturation stage, the remaining amelogenin is rapidly lost, and the enamel becomes fully mineralized. Proteinases in the enamel matrix are thought to be important in the digestion of amelogenins, but interaction of these proteinases with the developing enamel matrix is poorly defined.

Secretory enamel matrix has been reported to contain both metalloproteinases and serine proteinases (Shimizu *et al.*, 1979; Moe & Birkedal-Hansen, 1979; Crenshaw & Bawden, 1984; Carter *et al.*, 1984; Robinson *et al.*, 1984). The function of these proteinases in the enamel matrix is not known. Serine proteinases capable of hydrolysing amelogenins have been isolated in developing enamel (Shimizu *et al.*, 1979; Carter *et al.*, 1984, 1989). Studies by Overall & Limeback (1988) indicate that these serine proteinases appear in maturation-stage enamel.

Recent studies (Overall & Limeback, 1988; DenBesten & Heffernan, 1989) have shown the presence of multiple gelatinases, which are Ca^{2+} -dependent metalloproteinases, in the secretory enamel matrix. Overall & Limeback (1988) found that the gelatinases from pig secretory enamel with M_r values from 62000 to 130000 did not digest casein, amelogenin or albumin. However, earlier studies by DenBesten & Crenshaw (1987) showed caseinase activity in rat secretory enamel matrix that was inhibited by EDTA. This indicates that additional extracellular metalloproteinases active against casein are present in secretory-stage enamel.

Many connective tissues secrete neutral metallo-

proteinases in culture. According to a numbering system suggested by Okada *et al.* (1986), these include collagenase, or matrix metalloproteinase-1 (MMP-1), MMP-2, a gelatinase that degrades type IV collagen, and MMP-3, a proteoglycanase that degrades a number of macromolecules, including casein. Studies by Murphy *et al.* (1988) indicate that the metalloproteinase stromelysin described in fibroblast cultures (Wilhelm *et al.*, 1987; Murphy *et al.*, 1987), collagenase activator protein (CAP), which was found in articular cartilage (Treadwell *et al.*, 1986), and MMP-3 are the same proteinase.

In the present study we used an antibody to the MMP-3-like matrix metalloproteinase secreted by chondrocytes, collagenase activator protein (CAP), to identify this proteinase in the enamel matrix. The ability of this proteinase to digest amelogenins was determined.

MATERIALS AND METHODS

CAP antibody preparation

CAP was purified to a single band of M_r 57000 (determined by SDS/polyacrylamide-gel electrophoresis) from bovine-cartilage-conditioned medium as described by Treadwell *et al.* (1986). Monospecific polyclonal antiserum was prepared by injecting New Zealand White rabbits with an emulsion of Freund's complete adjuvant containing 200 μg of purified CAP. Three more injections of CAP in Freund's incomplete adjuvant were given at 2-week intervals. Serum was collected and screened for the presence of CAP antibody by Ouchterlony double-diffusion analysis. Specificity of the antibody was determined by immunoprecipitation of [^{35}S]methionine-labelled protein from bovine-articular-cartilage-conditioned medium.

Abbreviations used: MMP, matrix metalloproteinase; CAP, collagenase activator protein.

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Enamel preparation

Bovine enamel was obtained from the unerupted third molars of freshly slaughtered 1–2-week-old calves. The teeth were dissected from the alveolar bone, and the enamel organ was wiped from the teeth with cotton gauze dipped in ice-cold 0.9% NaCl solution. The soft secretory enamel was scraped from the teeth, pooled and stored at -80°C .

Western immunoblot

Enamel was demineralized in 5% (w/v) trichloroacetic acid for 30 min, centrifuged and dissolved to a final concentration of 2 mg of protein/ml in 50 mM-Tris/glycine buffer, pH 6.8, containing 0.1% SDS. Samples of reduced enamel protein and partially purified CAP were resolved by SDS/polyacrylamide-gel electrophoresis (10% gels), and electroeluted on to nitrocellulose in a Transblot apparatus (Hoeffer) for 60 min at 200 mA constant current with the use of the buffer system described by Towbin *et al.* (1979). The nitrocellulose filter was blocked with 3% (w/v) gelatin in 500 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.5, and treated with a 1:500 dilution of either rabbit anti-CAP serum or pre-immune serum. The filter was washed and treated with an immuno-gold-labelled goat anti-(rabbit IgG) second antibody (Bio-Rad Laboratories) and silver-enhanced according to the manufacturer's instructions.

Immunohistochemistry of bovine enamel

Unerupted third molars dissected from 2-week-old calves were immersed in 4% (w/v) paraformaldehyde for 48 h. The teeth were then demineralized in 22% (v/v) formic acid in 10% (w/v) sodium citrate for 3 weeks, embedded in paraffin and sectioned. The sections were deparaffinized, rehydrated and treated with H_2O_2 to destroy endogenous peroxidase activity. The sections were blocked with 10% (v/v) non-immune rabbit serum and incubated with a 1:300 dilution of either pre-immune serum or serum containing rabbit anti-CAP antibody. CAP was localized by incubating the sections with a biotinylated second antibody followed by a horseradish peroxidase conjugate (Zymed, San Francisco, CA, U.S.A.) according to the manufacturer's instructions. Sections were counterstained with haematoxylin and examined under the light microscope.

Proteinase digestion of amelogenin

Amelogenin was purified from secretory bovine enamel as follows. Enamel that had been demineralized for 3 days with 0.5 M-EDTA was dialysed against 50 mM-ammonium bicarbonate buffer, pH 8.0, freeze-dried and dissolved in 5 M-urea containing 2% (v/v) Ampholines pH 5–8. Enamel proteins were fractionated by preparative isoelectric focusing on a Rotofor cell (Bio-Rad Laboratories). Fractions containing the 27000- M_r amelogenin were pooled and refocused. Fractions that contained only the 27000- M_r amelogenin as determined by SDS/polyacrylamide-gel electrophoresis were combined, dialysed against 100 mM-ammonium bicarbonate buffer, pH 8.0, and freeze-dried. This purified amelogenin was labelled with fluorescein isothiocyanate as described by Twining (1984). The fluorescein isothiocyanate-amelogenin was used as a substrate to determine whether purified CAP would digest amelogenin.

A 10 μl portion of CAP (10 $\mu\text{g}/\text{ml}$ in 20 mM-Tris/HCl buffer, pH 7.2), isolated from cartilage-conditioned medium, was incubated for 18 h at 37°C with 10 μl of 0.1% fluorescein isothiocyanate-amelogenin in 50 mM-Tris/HCl buffer, pH 7.2, and 20 μl of 10 mM-Tris/HCl buffer, pH 7.8, containing 10 mM- CaCl_2 . A blank was prepared that did not contain CAP. The reaction was stopped by adding 120 μl of 5% (w/v) trichloroacetic acid. After 1 h the solution was centrifuged, and a 60 μl portion of the supernatant was diluted to 2 ml with 500 mM-Tris/HCl buffer, pH 8.6. Fluorescence was determined with the use of an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The difference between the relative fluorescence of the samples and their corresponding blank was determined. The activity of CAP against amelogenin was compared with the activity against fluorescein isothiocyanate-casein (Sigma Chemical Co.), determined by the same method.

RESULTS

The polyclonal antibody raised against CAP from bovine-cartilage-conditioned medium immunoprecipitated a single band on double diffusion against either crude cartilage-conditioned medium or the purified enzyme. The antibody immunoprecipitated a major band at M_r 57000 (CAP) from [^{35}S]methionine-labelled cartilage-conditioned medium (Fig. 1). Storage or various purification procedures or activation by *p*-aminophenylmercuric acetate resulted in other lower- M_r forms of the enzyme similar to those found by other investigators (Chin *et al.*, 1985; Murphy *et al.*, 1986).

A Western blot of freshly dissected enamel matrix proteins with the anti-CAP antibody showed that the antibody recognized a 57000- M_r and a 60000- M_r protein (Fig. 2). There was no antibody reaction when the blot was exposed to pre-immune serum (result not shown).

Immunohistochemistry showed specific staining in the

Fig. 1. Autoradiograph of biosynthetically labelled cartilage secreted proteins immunoprecipitated by pre-immune serum (lane A) or anti-CAP serum (lane B)

The positions of M_r standards are indicated on the left.

Fig. 2. Western-blot analysis of CAP in bovine cartilage and enamel

Lane A, partially purified CAP from cartilage-conditioned medium; lanes B and C, bovine enamel demineralized and precipitated with 5% trichloroacetic acid. The positions of M_r standards are indicated on the left.

enamel matrix (Fig. 3). Enamel near the dentine showed the strongest reaction with the antibody. Dentine matrix, particularly the area surrounding the odontoblastic tubules, reacted with the anti-CAP antibody and staining was increased at the dentino-enamel junction.

The assay for proteolytic activity of CAP against fluorescein isothiocyanate-amelogenin showed hydrolysis of amelogenin by CAP. However, activity against 27000- M_r amelogenin was low (4 relative fluorescence units) as compared with casein (18 relative fluorescence units). One relative fluorescence unit equals the fluorescence of a 50 μ M solution of quinine sulphate. Activity against both substrates was inhibited by EDTA.

DISCUSSION

In these studies an antibody was made to the metalloproteinase CAP (MMP-3, stromelysin), isolated from cartilage-conditioned medium. Immunoblots of EDTA-demineralized enamel protein showed specific cross-reactivity of the CAP antibody with protein in the enamel matrix. The antibody recognized the 57000- M_r proenzyme and a 60000- M_r protein, which was most probably the glycosylated form of the enzyme described by Wilhelm *et al.* (1987). Immunohistochemistry showed that this proteinase was localized in enamel, and that its presence in the enamel matrix was not due to contamination resulting from incomplete removal of the ameloblast cell layer during dissection.

Previous studies have shown that an important function of this proteinase is to activate procollagenase (Treadwell *et al.*, 1986; Murphy *et al.*, 1987; Ito & Nagase, 1988). The proteinase has also been shown to

Fig. 3. Immunohistochemistry of demineralized bovine enamel stained with a horseradish-peroxidase-conjugated second antibody to the anti-CAP antibody

(a) Sections incubated with anti-CAP serum showed staining in the enamel (E) and dentine (D) with intense staining along the dentino-enamel junction. The odontoblasts (OD) were also heavily stained. (b) Control sections showed no reaction after incubation with pre-immune serum.

be active against a wide range of substrates, including proteoglycan, gelatin, type IV collagen, laminin and fibronectin (Chin *et al.*, 1985; Okada *et al.*, 1986; Wilhelm *et al.*, 1987). We have shown that this proteinase is present in tissues such as enamel that do not contain collagen, and that amelogenin is also a substrate of CAP (stromelysin, MMP-3).

The relatively weak activity of CAP against amelogenin may be important in the slow hydrolysis of amelogenin that occurs during the secretory stage of enamel formation. Although hydrolysis of amelogenin begins immediately after secretion, rapid hydrolysis does not begin until the transition stage between secretory and maturation enamel. Absorption studies have shown that the initially secreted high- M_r amelogenin strongly inhibits crystal growth. The hydrolysed lower- M_r forms of amelogenin have a lower absorption affinity towards apatite crystals. This suggests that early mineralization may be controlled by a gradual degradation of amelogenins with a resulting loss of absorption affinity by the matrix constituents (Aoba *et al.*, 1987). A slow hydrolysis of amelogenins by a proteinase such as CAP, which is only slightly active against the amelogenins, could allow the elongation and growth of the early enamel crystals that form a crystal core for later rapid mineralization.

The hydrolysis of type IV (basement-membrane) collagen by stromelysin (Chin *et al.*, 1985; Wilhelm *et al.*, 1987) and MMP-3 (Ito & Nagase, 1988) in association with other gelatinases (MMP-2) suggests that CAP (stromelysin, MMP-3) may also play a role in cell turnover. A basement membrane separating the ameloblast cell layer from the enamel matrix is present during the stages of enamel development (transition and early maturation) when ameloblasts show morphological characteristics typical of absorbing cells (Takano, 1979). These proteinases may function in the rapid cell modulations and alterations in basement-membrane structures that occur during enamel development.

In conclusion, these results show that CAP, a matrix metalloproteinase that appears to be the same as MMP-3 or stromelysin, is present in the developing enamel matrix. The function of this proteinase in enamel development is not known, but it may be important in hydrolysing amelogenins or in the alterations in cell structure that occur throughout amelogenesis. In other tissues, such as cartilage, CAP appears to be part of a proteolytic cascade with a major function being the activation of collagenase. Further studies of other proteinases in enamel will show whether a similar cascade system is present in enamel.

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