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STUDIES ON THREE MATRIX MOLECULES IN BONE AND DENTIN

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Stockholm 2003

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Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-702-9

To Johan and Adam

ABSTRACT

The biomineralization of bone and dentin is a complex and dynamic process, involving the formation of an organic framework in which mineral crystals are deposited. The non-collagenous proteins (NCPs) of the organic phase play a central role in the extracellular matrix (ECM) organization and mineralization regulation. To facilitate the understanding of the mechanisms of biomineralization, it is crucial to expand our knowledge regarding the functions of the NCPs in this process. Therefore, this current study aimed to examine the protein composition of bone and dentin and to identify new components and elucidate their potential functions. Three principal matrix components were identified and subsequently investigated within this thesis; osteoadherin (OSAD), nucleobindin (Nuc) and calreticulin (CRT), and all appear to have a significant role in bone and dentin mineralization.

A combination of biochemical, morphological, cellular and molecular biological methods was used to characterize OSAD, Nuc and CRT within bone and dental tissues. OSAD was identified in extracts of bovine dentin and localized in odontoblasts, mineralized dentin matrix, cementum and surrounding alveolar bone, and ultrastructurally, it appeared to be associated with collagen fibrils. Nuc was detected within the tooth in odontoblasts, ameloblasts and dentin matrix, and within bone in the osteoid of newly formed bone, in compact bone and in the various bone cells. In addition, the role of Nuc during the mineralization process was investigated in rat calvaria-derived primary osteoblasts, grown under osteogenic conditions. Low levels of Nuc were detected during cell proliferation, which were subsequently upregulated during differentiation, nodule formation and initial mineralization, followed by a decrease in expression as the mineralization process proceeded. CRT was identified in extracts of bovine dentin and localized within odontoblasts, ameloblasts and, for the first time, extracellularly in predentin matrix. Additionally, to investigate potential functions of the proteins, an *in vitro* long bone organ culture system was established, using antisense oligonucleotides with the aim of knocking down gene expression and study possible effects on bone formation.

From the investigations performed herein, the three proteins studied appear to have significant functions during biomineralization. It could be speculated that OSAD might bind to collagen fibrils and possible act as a regulator of nucleation. Nuc might also play a role as a regulator in the biomineralization of bone and dentin. CRT, on the other hand, appears to play an important role in dentinogenesis and together with Nuc may contribute to the accumulation and transport of Ca^{2+} ions through the odontoblasts to the mineralization front before hydroxyapatite (HAP) deposition. In conclusion, the studies carried out within this thesis have contributed to the ever emerging picture of the role of NCPs in the dynamic formation of mineralized tissues.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-V)

I. Petersson U, Hultenby K and Wendel M (2003)
Identification, distribution and expression of osteoadherin during tooth formation
Eur J Oral Sci, Apr;111(2):128-36

II. Somogyi E*, Petersson U*, Sugars RV, Hultenby K and Wendel M
* Authors have contributed equally to this study
Nucleobindin - a Ca^{2+}- binding protein present in the cells and mineralized tissues of the tooth
accepted in *Calcif Tissue Int*

III. Petersson U, Somogyi E, Reinholt FP, Klinge B, Sugars RV and Wendel M
Nucleobindin is a potential regulator of mineralization in bone cells and extracellular matrix
submitted to *Bone*

IV. Somogyi E, Petersson U, Hultenby K and Wendel M (2003)
Calreticulin - an endoplasmic reticulum protein with calcium-binding activity is also found in the extracellular matrix
Matrix Biol, Apr;22(2):179-91

V. Petersson U, Sugars RV, Ganss B, Klinge B and Wendel M
The role of bone sialoprotein in endochondral bone formation
manuscript

TABLE OF CONTENTS

ABBREVIATIONS

BAG-75	Bone acidic glycoprotein-75
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CRT	Calreticulin
CS	Chondroitin sulfate
DMP-1	Dentin matrix protein-1
DPP	Dentin phosphoprotein
DS	Dermatan sulfate
DSP	Dentin sialoprotein
DSPP	Dentin sialophosphoprotein
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
HAP	Hydroxyapatite
KS	Keratan sulfate
LRR	Leucine-rich repeats
MEPE	Matrix extracellular phosphoglycoprotein
MGP	Matrix Gla Protein
MMP	Matrix metalloproteinase
NCP	Non-collagenous protein
Nuc	Nucleobindin
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
OSAD	Osteoadherin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Proteoglycan
PRELP	Proline, arginine-rich end leucine-rich repeat protein
PTH	Parathyroid hormone
PTHrP	PTH-related protein
RGD	One letter code for Arg-Gly-Asp
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SIBLING	Small Integrin-Binding LIgand, N-linked Glycoprotein
SLRP	Small leucine-rich proteoglycan
SPARC	Secreted protein, acidic, rich in cysteine
TGFβ	Transforming growth factor-β
TIMP	tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor

GENERAL INTRODUCTION

HARD TISSUES OF THE BODY

The hard tissues of the body comprise bone, cementum, dentin and enamel, and except for enamel, they share many similarities in composition and formation. Bone, cementum and dentin are all mineralized connective tissues, with collagen being the principal organic component, which, among other functions, plays a structural role (Robey, 1996). Common to the three tissues is the mesenchymal origin of the extracellular matrix (ECM) producing cells (Krebsbach and Robey, 2002). Moreover, the array of molecules synthesized by the different cells is similar, although not completely identical. In contrast, enamel is not a connective tissue, it contains no collagens and comprises a totally different range of enamel related molecules (Fincham *et al.*, 1999). In this thesis only bone and dentin will be discussed.

BONE

Bone is a mineralized and dynamic connective tissue with its major purpose to provide structural support, locomotion and protection of the body's internal organs. Furthermore, the skeleton plays a role in haematopoiesis and acts as a reservoir for ions involved in physiological mineral homeostasis (Karsenty, 2003).

Due to its gross appearance, bone can be classified as long or flat. Bones of the axial skeleton, such as tibia and femur, are long bones, and flat bones include the skull bones, mandible, scapula and pelvis. Microscopically, there are two distinct types of bone; woven, which is formed during growth or in response to injury, and lamellar bone, which is the load-bearing bone in the adult skeleton. Macroscopically, lamellar bone can be divided into compact and trabecular. Compact bone comprises the dense outer part, possessing mechanical and protective functions, and trabecular bone, also called spongy or cancellous bone, forms a network of bony partitions integrating the bone marrow cavity. The osteoid, synthesized by bone forming cells, is the unmineralized matrix, which will later mineralize.

Bone formation can occur by two different pathways. First, endochondral bone formation develops at the end of long bones, when cartilage tissues, formed by aggregated mesenchymal cells, are replaced by bone. The second type of ossification, intramembranous bone formation, occurs when mesenchymal cells directly differentiate into bone cells and produce a matrix of woven bone, which later mineralizes to form the flat bones (Karsenty, 1999).

The area responsible for virtually all longitudinal growth of the skeleton, mainly achieved by endochondral bone formation, is termed the epiphyseal growth plate (Figure 1), and it serves as a constant source of cartilage conversion to bone (Kronenberg, 2003). The growth plate is divided into distinct, but continuous zones, based on cellular size, distribution, activity and matrix constituents. The different regions are termed the reserve zone, the proliferative zone and the upper and lower

hypertrophic zone. Within the reserve zone the chondrocytes are rounded and appear to be arranged randomly in a matrix of collagen and proteoglycans (PGs). The cells eventually convert into a discoid form, organized in regular columns, characteristic for cells of the proliferative zone. Within the hypertrophic zones the cells become enlarged, attract blood vessels and chondroclasts, and subsequently undergo apoptosis, leaving behind a cartilage scaffold. This cartilage mould is used by the osteoblasts, derived from the bone marrow stromal cells, to settle and secrete ECM, which subsequently mineralizes (Kronenberg, 2003).

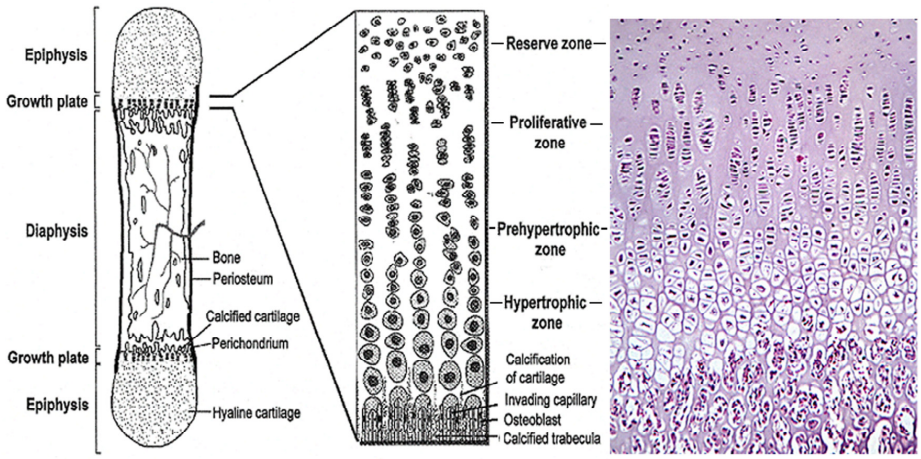


Figure 1. Schematical and histological illustration of the metaphyseal growth plate, showing the different regions. The histological image shows a section of a 5-day-old femur stained with haematoxylin-eosin.

BONE CELLS

A variety of bone cells have been identified to be involved in bone metabolism. The key cells during the process of bone formation are the ECM forming *osteoblasts*. Following ECM production, some osteoblasts become embedded in the osteoid and are subsequently named *osteocytes* and are responsible for the metabolism and maintenance of bone. The *lining cells* are inactive osteoblasts, which cover the bone surface in adult bone, and *osteoclasts* are responsible for the resorption of bone. *Stromal osteoprogenitor cells* contribute to the maintenance of the osteoblast population and bone mass.

Osteoblasts originate from mesenchymal osteoprogenitor cells of the bone marrow and periosteum (Aubin, 2001). Bone marrow stromal cells are a phenotypically heterogeneous population of mesenchymal precursor cells, which contribute to the regeneration of not only bone, but also to cartilage, adipose, muscle, and other connective tissues (Deans and Moseley, 2000; Mackie, 2003). Multipotent stem cells undergo successive stages of differentiation, giving rise to a number of committed progenitor and precursor cells, which in bone are the preosteoblasts. Proliferation and differentiation of the cells have been shown to occur under the influence of a number of transcription factors (such as Runx2/Cbfa1 and osterix), growth factors (such as transforming growth factor-β (TGFβ) and bone morphogenetic proteins (BMPs)) and hormones (such as parathyroid hormone (PTH) and PTH-related protein (PTHrP)) (Aubin, 2001). As a result, preosteoblasts differentiate into mature osteoblasts, which deposit a mixture of ECM proteins, including type I collagen as the main component, as well as non-collagenous glycoproteins, PGs, sialoproteins and phosphoproteins, which together with mineral form the mineralized matrix. Osteoblasts possess typical features of cells responsible for synthesis and secretion; they are polarized, contain a prominent rough endoplasmic reticulum (rER) and Golgi apparatus, abundant mitochondria, transport and secretory vesicles (Aubin, 2001).

As osteoblasts produce and secrete the collagenous and non-collagenous proteins (NCPs), several of them become embedded within the bone matrix, which later calcifies. These cells are termed osteocytes (Aarden *et al.*, 1994). The osteocytes are located in bone lacunae, connected to each other, to osteoblasts and lining cells through long cellular processes, which run in small canals of unmineralized matrix, called canaliculi. The cell processes have contact with each other via gap junctions. In contrast to osteoblasts, the osteocytes comprise relatively few organelles essential for matrix production and secretion, although a limited secretion of molecules may be necessary for osteocyte function. The osteocyte has been postulated to play a role in blood-calcium homeostasis and to act as a mechanosensory cell in the three-dimensional bone network, although the full importance of the osteocyte still remains to be elucidated (Burger *et al.*, 1995).

Bone is a dynamic tissue and undergoes constant remodelling, which means removal of old bone and replacement with new bone, with the key participant in this process being the osteoclast (Teitelbaum, 2000). The osteoclast is a multinucleated giant cell, which originates from the haematopoietic

mononuclear cells of the monocyte-macrophage lineage in the bone marrow. The mononuclear osteoclast precursors circulate in the blood and subsequently fuse to multinucleated cells on the bone surface. Osteoclastic differentiation has been shown to be regulated by two essential factors, secreted by osteoblastic cells; the tumor necrosis factor (TNF) related cytokine RANKL (receptor for activation of nuclear factor kappa B, also known as TRANCE), which is the ligand for the RANK receptor, and the growth factor colony-stimulating factor-1 (CSF-1) (Boyle *et al.*, 2003). In addition, a soluble decoy receptor, osteoprotegerin (OPG), has been identified. The discovery of the OPG / RANKL / RANK system has revealed a mechanism whereby preosteoblastic /stromal cells control osteoclastogenesis (Khosla, 2001). The binding between RANK, expressed on osteoclastic precursors, and its ligand RANKL has been shown to be critical for normal osteoclasts to form. OPG, on the other hand, binds to RANKL, thereby preventing RANKL from binding with RANK. As a result, OPG prevents the formation of osteoclasts and bone resorption. Consequently, in healthy bone, OPG helps to maintain the equilibrium of bone resorption and formation (Khosla, 2001).

Active osteoclasts attach to the bone surface in a region called the clear zone and create an acidic environment. Resorption is accomplished through secretion of protons and proteolytic enzymes, such as cathepsin K, in an area called the ruffled border. The osteoclasts degrade the exposed organic matrix in the resorption lacuna, and dissolved products are then engulfed and transported away in vesicles (Boyle *et al.*, 2003).

DENTIN

Dentin, the most abundant of the mineralized tissues that form the tooth is, as well as bone, a connective tissue. The study of dentinogenesis, as a model for the biomineralization process, possesses several important advantages compared to osteogenesis; dentin does not normally remodel, except from shedding, dentin is not involved in the Ca^{2+} homeostasis of the body, the participating regions, dentin, predentin and *odontoblasts*, are distinct and possible to dissect into relatively pure fractions (Linde and Goldberg, 1993).

Teeth develop on the first branchial arch and the cells involved are derived from both the oral ectoderm, which forms enamel-producing ameloblasts, and from the underlying ectomesenchyme, which form odontoblasts and cementoblasts (Thesleff and Nieminen, 1996). Initiation of odontogenesis occurs through signaling between the oral epithelium and underlying ectomesenchymal cell populations, and proceeds in different stages, termed after the morphology of the developing tooth germ: bud, cap and bell stage. The first morphological sign of tooth development is a thickening of the oral ectoderm, referred to as dental epithelium. At the bud stage, dental epithelium invaginates into the underlying ectomesenchyme. During cap stage, the epithelial bud continues to proliferate into the ectomesenchyme, which condensates. The condensed mesenchyme differentiates into the dental papilla, which gives rise to the tooth pulp and the

dentin-producing odontoblasts. Continued growth of the tooth germ leads to the bell stage, where the dental organ comes to resemble a bell, and subsequently dentinogenesis begins at late bell stage (Tucker and Sharpe, 1999).

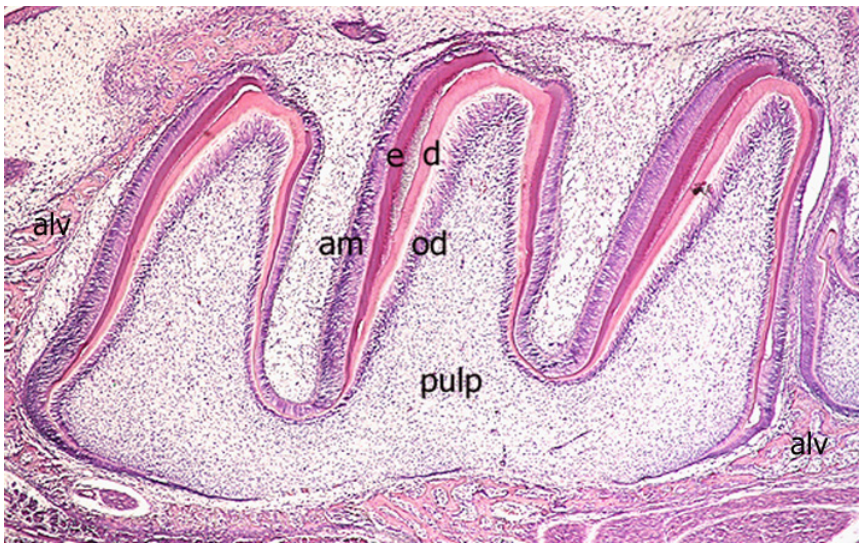
Initially during dentinogenesis, primary dentin is produced at a high rate during formation of the tooth. The first layer of primary dentin, deposited at the onset of mineralization, is called mantle dentin and the residual, main part of the primary dentin is termed circumpulpal dentin. After the tooth has erupted and become functional, the dentin forming cells synthesize secondary dentin on the pulpal side of the primary dentin, although this process of dentin formation is similar, but much slower. Tertiary, or reparative dentin is produced as a response to injury (Linde and Goldberg, 1993). Similar to bone, the tooth contains an unmineralized portion, the predentin, which is located between the odontoblasts and the mineralized dentin and remains throughout the life span of the tooth (Figure 2). The dentinal tubules, which run between the pulp and dentin, contain the odontoblastic processes.

ODONTOBLASTS

Odontoblasts, the cells of dentin, produce and secrete the organic components of the extracellular dentin matrix. Odontoblasts are neural crest derived cells that have differentiated from ectomesenchymal cells of the dental papilla (Ruch *et al.*, 1995). The differentiation of odontoblasts is characterized by a sequence of events, as first described by Ruch (Ruch, 1990). The process includes withdrawal from the cell cycle, cytological polarization and secretion of predentin/dentin. Differentiation is marked by the expression of several genes that encode for collagenous and NCPs. The terminal differentiation of odontoblasts has been shown to be controlled by the inner dental epithelium and the expression of signaling molecules, which interacts with the mesenchymal cells of the dental papilla. The most studied signaling molecules belong to the TGFβ, FGF (fibroblast growth factor), Shh (sonic hedgehog) and Wnt families. Odontoblast differentiation always starts from the tips of the cusps during bell stage of tooth morphogenesis (Ruch, 1998).

Furthermore, the transcription factor Runx2/Cbfa1 has been shown to be essential for odontoblast determination and differentiation (D'Souza *et al.*, 1999). The gene, restricted to dental mesenchyme during tooth formation, is regulated by epithelial signals. Runx2/Cbfa1 is expressed during cusp morphogenesis, but is downregulated at the onset of odontoblast differentiation. Runx2/Cbfa1 knockout mice die at birth and show a complete absence of osteoblasts and bone matrix (Komori *et al.*, 1997), while tooth development arrests at early bell stage, corresponding to the time of odontoblast differentiation (D'Souza *et al.*, 1999).

A



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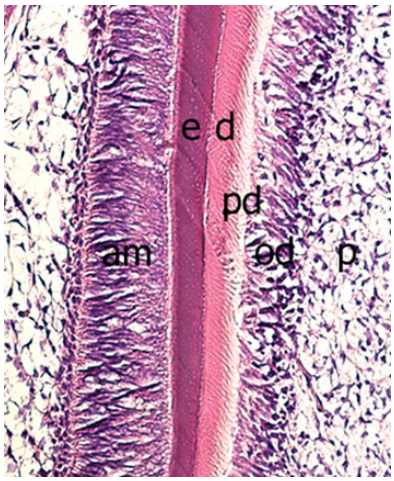

Figure 2. Histological section of a 5-day-old rat first molar stained with haematoxylin-eosin, showing the different constituents of a developing rat tooth.
(A) An overview of the first molar (B) A higher magnification of the various compartments of the growing molar.
p=pulp, od=odontoblasts, pd=predentin, d=dentin, e=enamel, am=ameloblasts, alv=alveolar bone

Prior to dentinogenesis, odontoblasts are small and undifferentiated, with a central nucleus, containing few cisternae of rER, only small Golgi apparatus and few mitochondria (Linde and Goldberg, 1993; Ruch, 1998). Influence from the cells of the internal dental epithelium, stimulates the mesenchymal cells of the dental papilla to initiate differentiation into larger preodontoblasts, which eventually become odontoblasts, as their cytoplasm increases in volume. Mature, secretory odontoblasts are tall, columnar, polarized cells, which possess the characteristics of a secretory cell, including a basally located nucleus, large cisternae of rER and a Golgi apparatus comprising secretory granules. Odontoblasts line the secreted predentin, with the cell body located within the pulp and the long odontoblastic process extending into the predentin/dentin matrix (Ruch *et al.*, 1995).

BIOCHEMISTRY OF BONE AND DENTIN

The biochemical composition of bone and dentin is similar, with a large inorganic mineral component and an organic phase. The inorganic phase comprises approximately 70% of the dry weight, and in dentin the value is somewhat higher compared to bone. The inorganic phase consists mainly of crystals of hydroxyapatite, and the crystals are embedded in the organic matrix. The organic matrix comprises mainly type I collagen (90% of the organic matrix), but also NCPs (10% of the organic matrix). In addition, the tissues also contain cells, lipids and water (Linde and Goldberg, 1993).

MINERAL PHASE

The mineral component of bone and dentin, composed mainly of a calcium phosphate salt, is closely related to naturally occurring crystalline calcium hydroxyapatite (HAP), $[Ca_{10}(PO_4)_6OH_2]$ (Robey and Boskey, 1996). In addition, the mineral phase of bone and dentin also contains small amounts of impurities, such as carbonate, citrate, sodium, magnesium and fluoride, which can replace the phosphate or hydroxyl groups and eventually change the physical properties of the crystals. The presence of other mineral phases in bone and dentin, for example brushite, amorphous calcium phosphate and octacalcium phosphate have been suggested as precursors to HAP, although the significance of these acidic calcium phosphate phases during the mineralization process is still not fully elucidated (Robey and Boskey, 1996).

ORGANIC PHASE

Bone and dentin both contain type I collagen as the main ECM component, with trace amounts of collagen type III, V and X (Linde and Goldberg, 1993). The NCPs identified in the two tissues are similar, although quantitative differences exist (Linde and Goldberg, 1993). Proteins common for both bone and dentin include bone sialoprotein (BSP), osteopontin (OPN), osteonectin (ON) and

osteocalcin. Furthermore, a number of PGs have been identified in bone and dentin, belonging to the family of small leucine-rich PGs (SLRP) (Embery *et al.*, 2001). Similar to bone, dentin contains growth factors (TGFβ) and serum-derived proteins, such as albumin and α_2-HS glycoprotein, which are brought to the region through the circulation, and have a high affinity for HAP (Linde and Goldberg, 1993). Moreover, dentin also possesses three proteins that initially were thought to be unique for the tissue; dentin phosphoprotein (DPP), which is the major dentin NCP, dentin sialoprotein (DSP) and dentin matrix protein-1 (DMP-1). DSP and DPP are encoded by a single gene, the dentin sialophosphoprotein gene (*dspp*). Lately, the genes coding for all the potential dentin specific proteins have been identified within bone, although in a much lower quantity (Qin *et al.*, 2002).

COLLAGENS

The collagens have been defined as "structural proteins of the ECM, which contain one or more domains harbouring the conformation of a collagen triple helix" (van der Rest and Garrone, 1991). The triple helix motif is composed of three chains with the repetitive sequence of Gly-X-Y, where X is often proline and Y hydroxyproline. The specific sequence aids in the coiling of each chain in a left-handed helix and the three chains together form a right-handed triple helix, stabilized by hydrogen bonds between the chains (Myllyharju and Kivirikko, 2001; van der Rest and Garrone, 1991).

Collagens comprise a family of 27 multimeric proteins, which are grouped in subfamilies depending on their structure and/or function; the fibrillar (types I, II, III, V and XI), non-fibrillar or basement membrane (types IV, VI, VII and XII) and fibril associated collagens with interrupted triple helices (FACIT) (types VIII, IX, X and XIII) (Myllyharju and Kivirikko, 2001).

The major collagen in mineralized ECM of bone and dentin is type I collagen, although small amounts of collagen types III, V and several FACITs have been identified. Type I collagen has been shown to be composed of two α1 chains and one α2 chain and the gene coding for the α1 chain of type I collagen has been located on human chromosome 17, while the gene for the α2 chain on chromosome 7 (Retief *et al.*, 1985). Type I collagen is synthesized by the osteoblast or odontoblast within the ER, where specific residues are hydroxylated. The propeptide, procollagen, possesses extensions at the N- and C-terminal, is secreted through the Golgi into the extracellular space. Three procollagen chains assemble in a triple helical structure in the central part, and the N- and C-termini subsequently become excised by specific matrix metalloproteinases (MMPs). Extracellularly, the mature processed collagen molecules aggregate to form larger collagen fibrils and help to form the ECM with other components (van der Rest and Garrone, 1991).

Electron microscopic studies of type I collagen fibrils give the impression that fibrils are striated, and this has been found to be the result of collagen molecules overlapping each other with a 40 nm gap between each consecutive

molecule. Studies have proposed that it is within these gap zones the initial mineral deposition begins. The collagen molecule itself has been shown to not act as a nucleator, but rather several of the NCPs have been reported to possess these properties.

The synthesis of type I collagen in bone and dentin producing cells, under both normal physiological and pathological conditions, have been shown to be regulated by a variety of molecules, such as growth factors (TGFβ, FGF), hormones (PTH, corticosteroids), cytokines (TNFα, interleukin-1) and vitamins (vitamin D), and the regulation appears to be mainly exerted at the transcriptional level. However, collagen production has been shown to be post - transcriptionally regulated (Ghosh, 2002). During the bone resorption process the degradation of the collagen molecule has been proposed to be commenced by a variety of enzymes, including MMPs (collagenases and gelatinises) and cathepsin K (Garnero *et al.*, 1998).

NON-COLLAGENOUS PROTEINS OF BONE AND DENTIN

The NCPs comprise approximately 10% of the organic phase of bone and dentin. They are synthesized either locally, in bone- or dentin producing cells, or they are generated elsewhere and transported via the circulation to the mineralized tissue. It is generally believed that the NCPs are involved in the organization of the matrix and the regulation of the mineralization process. The major NCPs of bone and dentin are listed in Table 1.

SIBLINGs (Small Integrin-Binding LIgand, N-linked Glycoprotein)

The SIBLINGs, a family of genetically related proteins, are products of five genes clustered along human chromosome 4 (Fisher *et al.*, 2001). The protein family includes bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1) and the recently discovered matrix extracellular phosphoglycoprotein (MEPE) (Rowe *et al.*, 2000). The classification of these proteins is not dependent on function, but rather on biochemical descriptions. BSP, OPN, DMP-1 and DSPP are similar in character, being acidic, phosphorylated, secreted proteins, although MEPE appears to be more distantly related in that it is not acidic. SIBLINGs bind strongly to HAP and furthermore, contain the integrin-binding RGD motif (Fisher *et al.*, 2001).

Table 1. **Non-collagenous proteins of bone and dentin**

Protein	Distribution and potential function
Bone sialoprotein	Present in mineralized tissues and during pathological mineralization Binds to HAP, binds cells via RGD-sequence Nucleator
Osteopontin	Expression not restricted to mineralized tissues Expressed during pathological mineralization Binds to Ca^{2+} and HAP, binds cells via RGD-sequence Inhibitor of nucleation Regulates osteoclast motility
Dentin phosphoprotein	Binds Ca^{2+} and phosphate ions, binds to collagen Nucleator and regulator of crystal growth, influencing crystal size and shape
Dentin sialoprotein	Present in dentin and in small amounts in bone Involved in epithelial-mesenchymal signalling during tooth development?
Dentin matrix protein-1	Expressed in bone and dentin Binds Ca^{2+} and HAP Nucleator, regulates transcription
MEPE	Expressed in bone and dentin Inhibitor of bone formation
Osteocalcin	Present in mineralized tissues Inhibitor of HAP crystal growth Specific marker for bone formation
Matrix Gla	Expression not restricted to mineralized tissues Potential inhibitor of mineralization
Osteonectin	Expression not restricted to mineralized tissues Binds Ca^{2+}, HAP and collagen
Bone acidic glycoprotein-75	Present in bone and cartilage Binds with high affinity for HAP, collagen and Ca^{2+} Nucleator

Bone sialoprotein (BSP)

BSP, a highly glycosylated and acidic phosphoprotein, possesses a high sialic acid content. The expression of BSP is restricted to mineralized tissues, where it is synthesized by osteoblasts, developing osteocytes, osteoclasts, hypertrophic chondrocytes, odontoblasts and cementoblasts (Ganss *et al.*, 1999). It has been reported that the promoter for BSP could be regulated by the transcription factor Runx2/Cbfa1 (Ducy *et al.*, 1997), which is known to be essential for osteoblast differentiation. Moreover, expression of BSP appeared to be stimulated by glucocorticoids, TGFβ and BMPs, but downregulated by vitamin D_3 (Ganss *et al.*, 1999).

Studies on the temporo-spatial expression and tissue localization of newly formed bone *in vivo* and *in vitro*, together with BSP's structural properties, have indicated that the protein might initiate HAP formation during *de novo* bone formation (Ganss *et al.*, 1999). BSP has been shown to be able to nucleate HAP formation in a steady-state agarose gel system under conditions of sub-threshold calcium phosphate supersaturation (Hunter and Goldberg, 1993). In support of the hypothesis that BSP might function as a nucleator, is the association of BSP with pathological mineralization, for example in breast tumours (Bellahcene *et al.*, 1994). Furthermore, BSP has been reported to facilitate a high binding affinity for Ca^{2+} and HAP (Chen *et al.*, 1992a). Transgenic mice have been produced, however no skeletal phenotype has been published.

BSP has also been shown to mediate cell attachment and spreading on plastic dishes (Somerman *et al.*, 1988), and an RGD-sequence has been identified close to the C-terminus of the protein (Oldberg *et al.*, 1988a). Cell attachment appeared to be mediated via the $\alpha_v\beta_3$ vitronectin receptor (Oldberg *et al.*, 1988b). However, it has also been reported that fragments of BSP, which do not contain the RGD motif were also able to mediate attachment of cells (Stubbs, 1996; Stubbs *et al.*, 1997), thereby indicating that BSP may be involved in alternate attachment mechanisms.

Osteopontin (OPN)

OPN, an anionic highly phosphorylated glycoprotein has been proposed to have a high affinity for HAP and Ca^{2+} (Franzen and Heinegard, 1985). The expression of OPN is not restricted to mineralized tissues, but has been detected in a number of tissues and biological fluids. Moreover, OPN synthesis has been observed in pathological conditions and pathophysiological responses, including, among others, atherosclerosis, various kidney diseases, cancer and in pathological mineralization such as kidney stone formation (Sodek *et al.*, 2000). In tooth, OPN has been detected mainly in cementum and mantle dentin, with very low amounts present in peritubular dentin (McKee *et al.*, 1996). The expression of OPN has been shown to be affected by several hormones (vitamin D and retinoic acid), cytokines and growth factors (epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGFβs, BMPs) (Denhardt and Noda, 1998). Different post-translational modifications exist, including phosphorylation, sulfation and glycosylation, which all have been shown to alter the structure of OPN.

OPN has been shown to be synthesized by and secreted from osteoblasts, osteocytes (McKee and Nanci, 1995), osteoclasts (Dodds *et al.*, 1995) and odontoblasts (Mark *et al.*, 1988). OPN, produced by osteoblasts, was shown to be enriched at surfaces where osteoclasts were bound to the bone matrix. OPN was first suggested to function as an anchor between mineral matrix of bone surfaces and osteoclasts during bone resorption (Reinholt *et al.*, 1990), although later, it was also proposed that the osteoclast itself was responsible for the synthesis of OPN into the resorption lacunas (Dodds *et al.*, 1995).

Furthermore, it has been shown that OPN binds, via its RGD-sequence, to the $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_9\beta_1$ and $\alpha_8\beta_1$ integrins, which mediate cell attachment, cell migration, chemotaxis, intracellular signaling and modulation of intracellular Ca^{2+} in several cell types, including osteoblasts and osteoclasts (Sodek *et al.*, 2000). OPN has been shown to be an extracellular ligand for CD44 (Weber *et al.*, 1996), a known cell surface marker for immune cells, but also expressed in the various bone cells, where it mediates cellular responses, similar to that of integrins, including adhesion, migration and stimulation of cells.

Several functions have been proposed for OPN, reviewed by Sodek et al., including the binding to mineral crystals in normal and pathological states, preventing nucleation and growth of HAP crystals, mediating cell attachment, migration and signaling through the RGD sequence, binding Ca^{2+} and HAP, acting as a chemoattractant for macrophages and preventing apoptosis (Sodek *et al.*, 2000). Interestingly, the skeleton of OPN-deficient mice have increased mineral content and crystal size in their long bones (Boskey *et al.*, 2002). OPN deficiency resulted in defected wound healing and osteoclast formation, and a downregulation of CD44 on the surface of the osteoclasts, which led to delayed and impaired bone resorption (Chellaiah *et al.*, 2003).

Dentin sialophosphoprotein (DSPP)

Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are expressed as a single mRNA transcript, coding for a large precursor protein, named dentin sialophosphoprotein (DSPP) (MacDougall *et al.*, 1997). The two proteins, DSP and DPP, have been shown to be transcribed as one transcriptional unit with a continuous open reading frame, which possesses the coding sequences for DSP at the 5´ end of the *dspp* gene and the sequences for DPP located at the 3´region (Feng *et al.*, 1998). Both DSP and DPP are found in the ECM of dentin, but DSPP, representing the entire protein sequence, has still not been identified in dentin ECM.

Until recently, the *dspp* gene and its coding proteins, DSP and DPP, were thought to be tooth specific, expressed only within dental tissues. However, using chemiluminescent Western immunoblot analysis and specific antibodies against rat DSP, the protein was detected in guanidine/EDTA extracts of rat long bone, although in much lower quantity (about 1/400), compared to the amounts of DSP present in dentin (Qin *et al.*, 2002). In addition, reverse transcriptase polymerase chain reaction (RT-PCR), using specific primers against DSP, DPP and the full

length DSPP, detected all three transcripts in cDNA from mouse calvaria, again in lower amounts compared to dentin (Qin *et al.*, 2002).

The *dspp* gene is localized on human chromosome 4 (MacDougall *et al.*, 1997) (mouse chromosome 5). This region also contains the critical loci for dentinogenesis imperfecta types II and III, and dentin dysplasia type II, which are diseases of teeth that display mineralization defects (Aplin *et al.*, 1995). The DSPP knock out mouse develops tooth defects similar to human dentinogenesis imperfecta type III, which is the most severe form of this disorder (Sreenath *et al.*, 2003). The symptoms reported included enlarged pulp chambers, widened predentin zone, hypomineralization, irregular mineralization front and pulp exposure (Sreenath *et al.*, 2003).

DPP, also called phosphoryn, is the most abundant NCP present in dentin, and has been proposed to play an important role during dentin mineralization (Butler, 1998). DPP is a highly phosphorylated protein, rich in phosphoserine and aspartic acid (Linde, 1988). The molecular weight of DPP has been described to vary between 65-140 kDa, depending on the degree of phosphorylation (Butler *et al.*, 1983). After synthesis by the odontoblasts (Munksgaard *et al.*, 1977), DPP has been shown to be transported to the mineralization front in a relatively short time (Linde, 1988). At the mineralization front, DPP has been ascribed several different important functions, including binding large amounts of Ca^{2+} and P_i ions (Marsh, 1989) and binding to type I collagen fibrils with high affinity (Traub *et al.*, 1992). Moreover, DPP has been suggested to act as a nucleator of HAP, and a regulator of crystal growth, influencing both crystal size and shape (Butler, 1998; Linde and Goldberg, 1993).

DSP, on the other hand, is a 53 kDa sialic acid-rich glycoprotein with an overall resemblance to other sialoproteins, including BSP, OPN and bone acidic glycoprotein-75 (BAG-75) (Butler *et al.*, 1992; Ritchie *et al.*, 1994). Lately, high molecular weight isoforms of DSP have been identified, with higher levels of carbohydrate modifications than originally reported (Qin *et al.*, 2003a). DSP has been immunolocalized to preameloblasts, odontoblasts, predentin, dentin (Butler *et al.*, 1992) and recently to bone (Qin *et al.*, 2002). The function of DSP is still not fully understood. The sequence of DSP has not revealed any cell binding RGD motif (Butler *et al.*, 1992), and DSP had little or no effect on *in vitro* HAP formation and growth of mineral crystals (Boskey *et al.*, 2000). However, localization of DSP in preameloblasts and young odontoblasts has suggested the possible involvement in some phase of epithelial-mesenchymal signalling during the developmental processes of the tooth (Butler, 1998).

Dentin matrix protein-1 (DMP-1)

DMP-1, a serine-rich acidic phosphoprotein, contains a single functional cell attachment RGD site and *N*-glycosylation sites (George *et al.*, 1993). Native DMP-1 possesses a molecular weight of 61 kDa, shown by Western blot analysis, and has been reported to be tightly bound to the mineral phase of dentin, being only extracted after demineralization (George *et al.*, 1993). Interestingly, the N-terminal region of DMP-1 is identical to that of BAG-75 (Srinivasan *et al.*, 1999).

DMP-1 was first considered as a dentin specific protein (George *et al.*, 1993), but has later been found in long bone and calvaria (MacDougall *et al.*, 1998). Full length DMP-1 cDNA has been cloned and sequenced from many different species (George *et al.*, 1993; Hirst *et al.*, 1997; MacDougall *et al.*, 1998), but the corresponding complete protein, including primary sequence with post-translational modifications such as glycosylation and phosphorylation sites, has not yet been isolated. Clusters of shorter fragments, which potentially have been shown to originate from DMP-1 have been identified in bone; a 37 kDa fragment originating from the N-terminal region, and a 57 kDa fragment originating from the C-terminal part (Qin *et al.*, 2003b).

RT-PCR has shown that transcripts for DMP-1 in developing mice molars appear at the late bud stage and levels then sustain throughout odontogenesis (D'Souza *et al.*, 1997). The expression pattern of DMP-1 in developing mouse teeth, shown by in situ hybridization, was detected in young odontoblasts before the onset of mineralization and the expression then decreased in secretory odontoblasts. This expression pattern was in contrast to DSPP, which co-localized with DMP-1 in young odontoblasts, but DSPP was then sustained at higher levels throughout mineralization (D'Souza *et al.*, 1997). In addition, another difference between expression patterns, was in that DMP-1 expression was detected in osteoblasts throughout skeletal ossification (D'Souza *et al.*, 1997). DMP-1 has also been identified in cementoblasts and ameloblasts during odontogenesis (MacDougall *et al.*, 1998).

Overexpression of DMP-1 in a preosteoblastic cell line, MC3T3-E1, and in pluripotent embryonic mesenchymal C3H101/2 cells, has shown differentiation of these mesenchymal cells to odontoblast-like cells occurs with regard to the expression of different transcription factors, such as Runx2/Cbfa1, BMP-2 and -4, different early ECM markers, such as alkaline phosphatase, OPN, ON, osteocalcin, and later markers, such as DSP (Narayanan *et al.*, 2001). Mineralized nodules were formed by the DMP-1 overexpressing cells, but when translation of DMP-1 was blocked by antisense expression, the initiation of the mineralization nodule formation in cell culture systems failed (Narayanan *et al.*, 2001), therefore suggesting a crucial role for DMP-1 during the mineralization process.

Matrix extracellular phosphoglycoprotein (MEPE)

MEPE (Rowe *et al.*, 2000), also known in the rat as OF45 or osteoregulin (Petersen *et al.*, 2000) is a recently discovered member of the SIBLING family. MEPE was found to be expressed by osteoblasts and odontoblasts, although the exact localization of the protein and the gene expression within bone and dentin tissues still remains to be clarified.

Deletion of the OF45 gene resulted in increased bone formation and bone mass (Gowen *et al.*, 2003). Cancellous bone histomorphometry revealed that the changed bone mass was the result of increased osteoblast number and osteoblast activity with unaltered osteoclast number, suggesting MEPE to play an inhibitory role in bone formation (Gowen *et al.*, 2003).

Osteocalcin

Osteocalcin, a major NCP synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes (Lian and Gundberg, 1988). It contains three residues of the vitamin K-dependent Ca^{2+}-binding amino acid γ-carboxyglutamic acid (Gla) (Vermeer, 1990). Osteocalcin is synthesized late during bone formation and thought to act as an inhibitor of HAP crystal growth (Romberg *et al.*, 1986). In addition, small amounts of osteocalcin have been shown to enter the blood stream, where it can be detected in serum, and used as a sensitive and specific marker for osteoblastic activity, correlated with bone formation (Brown *et al.*, 1984). The gene for osteocalcin has been determined on human chromosome 1 (Puchacz *et al.*, 1989).

Osteocalcin-deficient mice were shown to develop a phenotype marked by higher bone mass and bones of improved functional quality (Ducy *et al.*, 1996). The absence of osteocalcin also appeared to lead to an increase in bone formation, but without impairing bone resorption (Ducy *et al.*, 1996). In addition, dentin obtained from osteocalcin-deficient mice appeared to be structurally normal and did not show any marked differences in dentin matrix thickness and mineral content compared to wild type mice, suggesting that osteocalcin did not noticeably influence dentinogenesis (Bronckers *et al.*, 1998). The influence of compensatory mechanisms from other Gla containing molecules should not be excluded.

Matrix Gla Protein (MGP)

MGP, first isolated in 1983, contains five residues of the vitamin K-dependent Ca^{2+}-binding amino acid Gla and three phosphoserines (Price *et al.*, 1983). MGP has been detected in bone, dentin, cartilage (Robey, 1996), and moreover, in the vascular system, developing kidney, heart, and lungs (Fraser and Price, 1988). Overexpression of MGP decreased mineralization *in vitro* and also delayed chondrocyte maturation, suggesting that MGP might act as an inhibitor of mineralization (Yagami *et al.*, 1999). MGP has also been linked to pathological calcifications. Studies have shown that MGP-deficient mice die within two months of birth, due to arterial calcification and subsequent rupture of blood vessels. Interestingly, these mice have also displayed inappropriate calcification of cartilage (Luo *et al.*, 1997).

Osteonectin (ON)

ON, also named SPARC (secreted protein, acidic, rich in cysteine) and BM-40, is the most abundant glycoprotein produced by osteoblasts in developing bone (Termine *et al.*, 1981b). In addition, ON has been shown to be present in dentin in considerable amounts (Termine *et al.*, 1981b). The name osteonectin originates from the molecule's ability to bind Ca^{2+}, HAP and collagen, and to nucleate HAP deposition (Termine *et al.*, 1981b). ON contains two EF hand high affinity Ca^{2+} - binding domains (Maurer *et al.*, 1992; Yan and Sage, 1999) and has been demonstrated to have Ca^{2+} - binding functions both extracellularly and intracellularly (Busch *et al.*, 2000). Moreover, ON is found in a variety of tissues, both mineralized and non-calcifying, although its concentration is relatively low in

tissues other than bone and dentin. Several functions for ON have been proposed, including inhibition of cell proliferation, stimulation of metalloproteinase expression and regulation of matrix assembly. In addition, ON can bind to and inhibit the function of TGFß and other growth factors (Bradshaw and Sage, 2001).

ON-null mice were shown to develop a bone phenotype similar to osteopenia, including decreased bone formation and a reduction of osteoblast and osteoclast number, leading to reduced bone remodeling with a negative bone balance (Delany *et al.*, 2000). Moreover, collagen fibrils in the skin of ON-null mice were shown to be small and uniform in diameter, in contrast to normal mice, where fibrils possessed a variety of diameters (Delany *et al.*, 2000).

Bone acidic glycoprotein-75 (BAG-75)

BAG-75, an acidic phosphorylated glycoprotein was first isolated from rat calvaria and since then, detected only in bone and cartilage (Gorski and Shimizu, 1988). BAG-75 has been reported to possess a high affinity for HAP, collagen and Ca^{2+} (Chen *et al.*, 1992b) and together with BSP, participate in the nucleation and growth of apatite crystals during mineralization (Gorski *et al.*, 1997). Interestingly, the absolute structure of this protein remains to be characterized, since no one has successfully completely sequenced it, and furthermore, the gene locus is also unknown.

Serum proteins

α_2HS-glycoprotein, produced in the liver, is the most abundant serum protein in mineralized bone (Triffitt *et al.*, 1976) and dentin (Takagi Y *et al.*, 1990). α_2HS-glycoprotein is the human analogue of bovine fetuin and has been shown to bind to both HAP and Ca^{2+}. Moreover, fetuin was shown to bind to several members of the TGFβ superfamily, including TGFβ-1 and -2 and BMP-2, -4 and -6 (Demetriou *et al.*, 1996). A potential function for fetuin as an inhibitor of apatite formation in mineralized tissues has been proposed (Schinke *et al.*, 1996). Interestingly, elevated levels of serum fetuin correlates with a common form of mild osteogenesis imperfecta (Dickson *et al.*, 1983), whereas depressed levels are observed in Paget's disease, a condition characterized by increased bone turnover (Ashton and Smith, 1980).

Albumin, another serum protein produced in the liver, is also concentrated in bone several fold to levels found in the circulation (Triffitt and Owen, 1977). The function of albumin in mineralized tissues is not fully understood, however, *in vitro* studies have shown that albumin inhibits HAP growth by binding to the crystal, and inhibit crystal aggregation (Garnett and Dieppe, 1990).

SMALL LEUCINE-RICH PROTEOGLYCANS (SLRP)

The SLRPs belong to an expanding family of structurally similar, but genetically distinct molecules (Iozzo, 1999). Currently, 13 members of the SLRP family have been identified within extracellular matrices (Ameye and Young, 2002), and several of them have also been identified in predentin and dentin (Embery *et al.*, 2001). The SLRPs have been characterized by a small leucine-rich protein core of approximately 40 kDa, composed of three distinct domains (Iozzo, 1999). The N-terminal domain contains the negatively charged and covalently attached glycosaminoglycan (GAG) chains, and the central domain of the protein comprises approximately 80% of the molecule, composed of tandem repeats of leucine-rich regions. This domain is believed to fold in a similar horseshoe manner as the leucine-rich ribonuclease inhibitor, leaving the concave face of the protein to interact with other molecules (Kobe and Deisenhofer, 1995). The C-terminal contains a number of cysteine residues that form a loop.

Based on structural similarities, the SLRPs have been grouped into three main classes. The first class includes decorin (Krusius and Ruoslahti, 1986), biglycan (Fisher *et al.*, 1989; Neame *et al.*, 1989) and asporin (Henry *et al.*, 2001; Lorenzo *et al.*, 2001). The second class comprises lumican (Blochberger *et al.*, 1992), fibromodulin (Oldberg *et al.*, 1989), keratocan (Corpuz *et al.*, 1996), proline, arginine-rich end leucine-rich repeat protein (PRELP) (Bengtsson *et al.*, 1995) and osteoadherin/osteomodulin (Sommarin *et al.*, 1998). The third class within the SLRP family contains epiphycan/proteoglycan-Lb (Shinomura and Kimata, 1992), mimecan/osteoglycin (Madisen *et al.*, 1990) and opticin/oculoglycan (Friedman *et al.*, 2000; Reardon *et al.*, 2000). Chondroadherin (Neame *et al.*, 1994) and nyctalopin (Bech-Hansen *et al.*, 2000; Pusch *et al.*, 2000) do not belong to any of the described classes, but are closely related to each other. In Table 2, the class I and II members of the SLRP family are listed.

Decorin

Decorin is the archetypal class I member of the SLRP superfamily. The core protein has a molecular weight of 38 kDa and contains 11 leucine-rich repeats (LRR) and three potential glycosylation sites, of which only one is substituted with GAG (Krusius and Ruoslahti, 1986). The nature of the attached GAG chain appears to vary depending on tissue distribution. Within mineralized tissues, decorin has been described to be substituted primarily with chondroitin sulfate (CS), whereas in soft connective tissues the dermatan sulfate (DS) GAG chain appears to predominate (Robey, 2002). The decorin gene is located on human chromosome 12 (mouse chromosome 10) and is encoded by eight exons (McBride *et al.*, 1990).

Decorin has been identified in a variety of tissues, including bone (Fisher *et al.*, 1989) and teeth (Rahemtulla *et al.*, 1984). Within the tooth, decorin has been located in dental papilla cells, preodontoblasts and in the Hertwig´s epithelial cells. Furthermore, decorin was present along the calcification front, in the dentinal tubules and in odontoblast cell bodies with their processes in (Yoshiba *et al.*, 1996). In developing bone, decorin has been immunolocalized to the osteoid matrix and within osteoblasts (Bianco *et al.*, 1990) and in more mature bone, decorin was

found in association with osteocyte lacunae and within canaliculi of osteocytes (Ingram *et al.*, 1993).

The function of decorin in mineralizing tissues has been extensively studied and it has been shown to bind to collagen and regulate collagen fibrillogenesis (Vogel *et al.*, 1984). Furthermore, decorin has been shown to bind Ca^{2+} and interact with HAP and regulate crystal growth (Sugars *et al.*, 2003). Decorin also binds to TGFβ and regulates its activity and availability (Yamaguchi *et al.*, 1990).

Targeted deletion of the decorin gene resulted in a phenotype similar to the human Ehlers-Danlos syndrome, a clinically and genetically heterogeneous connective tissue disorder characterized by skin hyperextensibility, joint hypermobility, and tissue fragility (Danielson *et al.*, 1997). Moreover, abnormal collagen fibril morphology, including changes in fibril size, shape and orientation were observed, although no major phenotypic changes in bone and teeth were reported (Ameye and Young, 2002).

Table 2. **Small leucine-rich PGs in bone and dentin**

Protein	Distribution and potential function
Decorin	Expression not restricted to mineralized tissues Binds to collagen and regulates collagen fibrillogenesis Binds Ca^{2+}, HAP, TGFβ
Biglycan	Expression not restricted to mineralized tissues Binds collagen, TGFβ and HAP
Asporin	Expressed in various tissues, including bone, cartilage, teeth, heart and liver
Lumican	Major component of the cornea, but also in skin, muscle, cartilage, bone and dental tissues A potential marker for osteoblasts differentiation Binds to collagen and regulates collagen fibrillogenesis
Fibromodulin	Expression not restricted to mineralized tissues Binds to collagen and retards fibrillogenesis Binds to TGF-β
Osteoadherin	Expressed in bone and tooth Promotes integrin ($\alpha_v\beta_3$)-mediated cell binding Binds HAP

Biclycan

Similar to decorin, biglycan is another class I member of the SLRP family. The core protein has a molecular weight of 37 kDa and it has been shown to contain 12 LRR. In contrast to decorin, biglycan is substituted with two GAG chains, which in mineralized tissues mainly comprises CS and in non-mineralized tissues contains DS (Fisher *et al.*, 1989). The gene has been localized to the human X chromosome and has been shown to code for eight exons (McBride *et al.*, 1990). Interestingly, biglycan shows a 55% sequence homology to decorin (Fisher *et al.*, 1989).

Within tooth, biglycan has been immunolocalized in young functional odontoblasts and in both predentin and dentin. Interestingly, at an ultrastructural level it has been reported that biglycan showed a constant level across predentin and dentin, whereas labeled antibodies for decorin demonstrated a gradient distribution, with a three-fold increase in the distal predentin (Septier *et al.*, 2001). Furthermore, in bone tissue, decorin and biglycan have shown different distributions. Biglycan has been detected throughout the bone matrix in mature bone (Ingram *et al.*, 1993), and in developing bone it was found to be associated with the walls of the osteocyte lacunae and the bone cell surface, thus indicating different functions for biglycan and decorin, due to their distinct tissue distribution (Bianco *et al.*, 1990).

Proposed functions for biglycan include binding to type VI collagen (Wiberg *et al.*, 2001). Biglycan also possesses the capacity to bind to TGFβ and HAP (Robey, 2002).

Deletion of the biglycan gene in mice leads to an osteoporosis-like phenotype (Xu *et al.*, 1998), including reduced growth rate and decreased bone mass with age, due to lower osteoblast number and activity and a decreased number of bone marrow stroma cells with age (Chen *et al.*, 2002). In addition, knockout mice showed irregular and a marked diameter variability in collagen fibril size and shape (Ameye and Young, 2002). Interestingly, decorin/biglycan double knockout mice have been produced to study possible compensatory effects of the respective molecules. The decorin/biglycan double knockout showed a more severe bone phenotype than the biglycan single-deficient mouse (Ameye and Young, 2002), which suggests that biglycan and decorin, although possessing distinct functions, are still sufficiently similar to compensate their absence in the singly deficient mice.

Asporin

Asporin, the most recent member of the SLRP-family to be detected, was purified from human articular cartilage and meniscus (Lorenzo *et al.*, 2001) and concurrently identified in full-length mouse cDNA by genetic approach (Henry *et al.*, 2001). During mouse embryonic development, the expression of asporin in different tissues was shown to be similar to that of decorin and biglycan, with high mRNA expression levels in the skeleton and other specialized connective tissues, including the tooth region (Henry *et al.*, 2001). However, the presence of asporin during osteogenesis and odontogenesis has not been further investigated.

Lumican

Lumican, a class II member of the SLRP family, and in contrast to the class I members, is substituted with keratan sulfate (KS) in the LRR region (Blochberger *et al*., 1992). The core protein has a molecular weight of 38 kDa and contains five potential N-glycosylation sites and 11 leucine-rich motifs (Blochberger *et al.*, 1992). The gene for lumican has been localized on human chromosome 12 (mouse chromosome 10), and contains three exons and two introns (Chakravarti *et al.*, 1995).

Lumican is a major component of the corneal stroma, although it has also been found in other connective tissues, such as skin, muscle, cartilage, bone matrix and dental tissues (Raouf *et al*., 2002). Interestingly, lumican has been suggested to be a potential marker for osteoblast differentiation (Raouf and Seth, 2002). Microarray analysis identified bone-related genes in osteoblast development, showed lumican expression to be absent during proliferation of MC3T3-E1 (a mouse calvaria-derived cell line), but strongly upregulated during differentiation and mineralization. Lumican was immunolocalized to cartilage during early mouse embryonic development, although in older embryos, lumican was found mainly within the newly forming bone matrix and in smaller amounts in cartilage (Raouf *et al*., 2002). In the tooth, lumican has been immunolocalized in predentin and to a lesser extent in dentin, pulp (Hall *et al.*, 1997) and cementum (Cheng *et al.*, 1996).

Deletion of the lumican gene was reported to cause skin fragility and corneal opacity (Chakravarti *et al*., 1998). Moreover, lumican deficiency resulted in severe effects on collagen fibrils structures, including thicker fibrils and an unorganized and irregularly spaced collagen fibril matrix, indicating an important role for lumican during fibrillogenesis (Chakravarti *et al*., 1998). No bone or dentin phenotype has been published, although biomechanical tests have revealed that the long bones are stronger but brittle compared to wild types (Chakravarti and Jepsen, unpublished observations in (Chakravarti, 2002)). Due to lumican's distinct expression pattern in mature osteoblasts and the known function to regulate size and spacing of collagen fibrils in the cornea, the suggested role for lumican in bone and dentin may be in the organization of the collagen network prior to mineral deposition (Hall *et al.*, 1997; Raouf *et al.*, 2002).

Fibromodulin

Fibromodulin was first identified as a 59 kDa protein in cartilage (Heinegard *et al.*, 1986), but has since been detected in sclera, tendon, bone matrix and in dental tissues (Cheng *et al.*, 1996; Gori *et al.*, 2001; Nurminskaya and Birk, 1996). The core protein of fibromodulin shares a 47% sequence homology to lumican, it contains 10 leucine-rich motifs, possesses five potential N-glycosylation sites and is substituted with KS chains (Plaas *et al.*, 1990). The human fibromodulin gene has been localized to chromosome 1 (mouse chromosome 1) and similar to lumican, contains three exons (Antonsson *et al.*, 1993).

Fibromodulin binds to fibrillar collagens and retards fibrillogenesis *in vitro* (Hedbom and Heinegard, 1989). Interestingly, fibromodulin and lumican compete for the same binding site on type I collagen fibrils (Svensson *et al.*, 2000), which is

distinctly different from that of decorin (Hedbom and Heinegard, 1993). In addition, both native and recombinant fibromodulin have been shown to bind TGFβ, indicating that fibromodulin may regulate TGF-β activities by sequestering TGF-β into the ECM (Hildebrand *et al.*, 1994).

The fibromodulin-null mice have been reported to have abnormal and fewer collagen fibril bundles, with the fibrils being thinner and more irregular (Svensson *et al.*, 1999). The mice have been shown to suffer from ectopic ossification of tendons and to develop osteoarthritis (Ameye *et al.*, 2002). Interestingly, the expression of lumican was shown to be four-fold upregulated in the fibromodulin-null mice in comparison to wild-type mice, adding further confirmation that lumican binds to the sites normally used by fibromodulin (Svensson *et al.*, 1999). A study by Svensson et al. later confirmed that lumican and fibromodulin do compete for the same binding sites on collagen fibrils (Svensson *et al.*, 2000).

Osteoadherin (OSAD)

OSAD, a KS containing SLRP, was first isolated from mineralized bovine long bone by Wendel et al. (Wendel *et al.*, 1998). The entirely translated bovine primary sequence of OSAD corresponds to a 49 kDa protein, which demonstrates high sequence homology to human, mouse and rat OSAD (Sommarin *et al.*, 1998). Moreover, bovine OSAD shows high sequence homology to the other SLRP class II members, including bovine keratocan (42% sequence homology) bovine fibromodulin and lumican, and human PRELP (37-38% identity) (Sommarin *et al.*, 1998). The central region of OSAD contains 11 B-type, leucine-rich repeats, ranging in length from 20 to 30 residues (Figure 3) (Sommarin *et al.*, 1998), and additionally, OSAD possesses a large and very acidic C-terminal domain, which is unique for OSAD amongst the other SLRP class II members (Sommarin *et al.*, 1998). OSAD promotes integrin ($\alpha_v\beta_3$)-mediated cell binding and binds strongly to HAP (Wendel *et al.*, 1998). OSAD has been shown to possess six tyrosine sulfates at the N-terminal region and two additional sulfated tyrosine residues close to the C-terminus (Onnerfjord *et al.*, 2003).

OSAD has been immunolocalized to the primary spongiosa of bovine fetal rib growth plate (Wendel *et al.*, 1998) and a recent study also reported OSAD to possess a similar ultrastructural distribution to BSP in rat long bone and calvaria (Ramstad *et al.*, 2003). Ultrastructural immuno-labelling for OSAD was localized in the mineralized bone matrix, with the highest concentration at the border between bone and cartilage (Ramstad *et al.*, 2003). Furthermore, mRNA expression of OSAD has been detected, by *in situ* hybridization, in osteoblasts on the surface of bone trabeculae, and the expression was found to be especially strong at the interface between cartilage and bone. Northern blot analysis of RNA samples from various bovine tissues, including brain, spleen, kidney, liver, heart, skeletal muscle, skin, bone marrow, tendon and

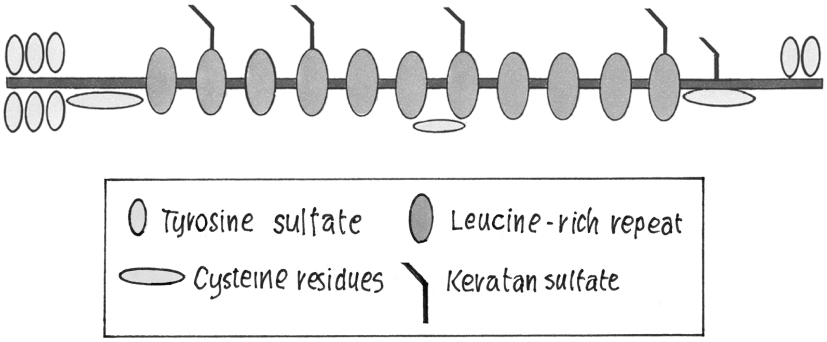

Figure 3. Structural features of OSAD. The molecule consists of a central domain of 11 leucine-rich repeats, six tyrosine sulfate sites at the N-terminal and two at the C-terminal. OSAD contains four cysteine residues at the N-terminal, two in the central LRR region and two in the C-terminal end, and the core protein is substituted with KS GAG chains.

bone showed a 1.9 kb transcript for OSAD to be expressed in bone, tendon and bone marrow only (Shen *et al.*, 1999). Lately, OSAD has been detected in human and rat developing teeth (Buchaille *et al.*, 2000). Specific expression for OSAD, identified by *in situ* hybridization, was detected in human mature odontoblasts of an extracted pulp from a third molar tooth germ. Weak OSAD expression was also detected in pulp cells (Buchaille *et al.*, 2000). Furthermore, in developing rat teeth, expression for OSAD was first detected in the surrounding alveolar bone, in sections from 19-day-old embryos (Buchaille *et al.*, 2000), and in newborn rats, transcripts for OSAD were detected in secretory ameloblasts and odontoblasts with a weaker signal identified in young ameloblasts, odontoblasts and in pulp. In 2-day old rats, an upregulation of OSAD expression in the mentioned cells was also reported (Buchaille *et al.*, 2000).

An *in vitro* human tooth slice culture system has reported the synthesis of OSAD by mature odontoblasts to be stimulated by TGFβ-1, following immunohistochemistry with an antibody against OSAD (Lucchini *et al.*, 2002).

The function of OSAD in mineralized tissues has still not been fully established, however, data indicates that OSAD might play a role as a regulator of mineralization. Potential functions of OSAD during the mineralization process of bone and dentin will be discussed later within this thesis.

REGULATION OF MATRIX PROTEINS

Remodeling of the bone extracellular matrix is an important aspect of the mineralization process. Several genes have been described to regulate the expression of matrix proteins, such as Runx2/Cbfa1, Dlx5, Msx1 and 2, osterix, members of the Wnt family and Twist, and a small number of these genes have been suggested to act as "master genes", capable of controlling and coordinating the expression of many matrix genes (Young, 2003). For instance, Runx2/Cbfa1 knockout mice have been reported to die just after birth and were incapable of forming bone due to a total lack of osteoblasts (Komori *et al.*, 1997). Furthermore, osteocalcin and OPN promoters have been shown to require Runx2/Cbfa1 for protein synthesis (Ducy *et al.*, 1997). Recently, another "master gene" candidate was identified, termed osterix (Nakashima *et al.*, 2002). Similar to Runx2/Cbfa1 knockout mice, no bone formation occurred in the osterix-null mice and several bone matrix proteins failed to be synthesized (Nakashima *et al.*, 2002). Osterix was shown to act downstream of Runx2/Cbfa1, and the expression was induced by BMP-2 (Yagi *et al.*, 2003).

Matrix metalloproteinases (MMPs)

Another class of molecules involved in normal extracellular matrix remodeling and bone growth are the MMPs. MMPs can be divided into subgroups, including the collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), membrane-type MMPs (MT-MMPs) (MMP-14, -15, -16, -17, -24 and -25) and other MMPs (MMP-7, -12, -19, -20, -21, -22, -23, -26, -27 and -28) (Vu and Werb, 2000). Common for all the MMPs, are that they are active at a neutral pH, require Ca^{2+} for activity and contain Zn^{2+}. MMPs are regulated at several levels, including post-translational modifications, inactivation by specific tissue inhibitors of metalloproteinases (TIMPs) or activation by pathways such as the plasminogen activator/plasmin pathway. The activated MMPs are then able to degrade collagens and other ECM proteins.

A variety of MMPs have been implicated in hard tissue formation and remodeling. In dentin formation, it has been established that following predentin secretion, the tissue undergoes remodeling prior to HAP deposition, and various MMPs have been implicated for this process (Caron *et al.*, 1998). MMP-3, also termed stromelysin-1 or proteoglycanase, capable of degrading the core protein of PGs, has been immunolocalized in predentin in a reverse distribution pattern in comparison to CS-substituted PGs, suggesting involvement of this specific MMP in the modification of CS PGs before mineralization (Hall *et al.*, 1999). In contrast, lumican, a KS-containing PG, was also found to be highly present in this area, which implied that MMP-3 degraded CS PGs in favor of KS PGs and thereby indicated a potential function as initiators of mineralization (Hall *et al.*, 1999).

Growth factors in bone and dentin

Within the mineralized tissues, bone and dentin, several growth factors, such as members of the TGFβs family, including TGFβ isoforms, activins and inhibins, growth differentiation factors and BMPs. Furthermore, FGFs have been identified as present in mineralized tissues.

Transforming growth factor β-1 (TGFβ-1)

TGFβ-1, a prototype of the TGFβ superfamily, is a multi-functional growth factor expressed in a wide variety of developing tissues from the early stages. TGFβ-1 is activated via the Smad signal transduction pathway and is a potential regulator of cell growth and differentiation. TGFβ-1 also regulates the synthesis of bone and dentin matrix proteins, by the stimulation of several genes but inhibition of others. Moreover, TGFβ inhibits matrix degradation by increasing the production of protease inhibitors and decreasing the production of proteases.

Investigations have reported stimulatory effects of TGFβ-1 on cells of mesenchymal origins, and inhibitory effects for cells of epithelial or neuroectoderm origin (Massague, 1990). During odontogenesis, TGFβ-1 was expressed by preodontoblasts and odontoblasts and the expression pattern appeared to coincide with the expression of type I collagen, thereby suggesting a role for TGFβ-1 in the initiation of collagen synthesis and cytodifferentiation of odontoblasts (Kitamura and Terashita, 1997). It has also been shown that TGFβ-1 downregulates DMP-1 and DSPP in odontoblasts and all key components of the TGFβ-1 signal transduction pathway (Smads 2, 3 and 4 and the specific TGFβ-1 type I receptor ALK5) are expressed by odontoblasts (Unterbrink *et al.*, 2002).

TGFβ-1 null mutation mice shows unaffected tooth initiation, morphogenesis and cytodifferentiation (D'Souza and Litz, 1995). However, with time the TGFβ-1 null mice showed loss of dental hard tissues through gradual destruction or wearing of both the enamel and dentin and also the degree of calcification was decreased (D'Souza *et al.*, 1998). In addition, it should also be noted that mineralization defects appeared in the bones and the mice were smaller than the wild type mice.

Bone morphogenetic proteins (BMP)

The BMPs are part of the large multigenic TGFβ superfamily, and to date, more than 40 BMPs have been identified (Saku, 1998). Similar to TGFβ-1, the BMPs are activated via the Smad signal transduction pathway.

Several BMPs (BMP-2, 4-7 and -9) show osteogenic capacity. In particular, BMP-2 has been revealed to be a key molecule in bone metabolism. Subcutaneous injection in rats of recombinant BMP-2 resulted in an induction of ectopic bone formation (Wozney *et al.*, 1988). Furthermore, BMP-2 has been shown to possess the capacity to differentiate progenitor cells into the osteoblastic lineage (Rosen *et al.*, 1996). Clinically, it has been demonstrated that BMP-2 facilitates repair and/or regeneration of bone defects and reverses osteopenia, if systemically administered (Bostrom *et al.*, 1999). A recent study identified BMP-2 responsive genes, by microarray gene expression, during cell (C2C12 myoblast cell

line) differentiation into the osteoblast lineage, after treatment with BMP-2 for 24 h (Balint *et al.*, 2002). An initial response of BMP-2 (after 2h) was detected in nuclear proteins and developmental regulatory factors, including Runx2/Cbfa1 and several homeobox proteins. After 8h, an induction of signaling proteins and ECM proteins was detected, such as type I and VI collagens and the members of the SLRP family, including osteoadherin. A bone phenotype was further established after 16h, by induction of genes for cell adhesion, communication and also enzymes implicated in bone ECM organization. Taken together, this study strongly supports the evidence for BMP-2 as an important molecule in osteoblast differentiation (Balint, 2003).

Ca^{2+} TRANSPORT IN BONE AND DENTIN

When a mineralized tissue, such as bone or dentin is formed, Ca^{2+} and P_i ions need to be transported from the vascular system across the cell layer, to become incorporated into the mineral phase at the very mineralization front. Most early studies concerning the role of Ca^{2+} in osteogenesis and dentinogenesis investigated Ca^{2+} as intracellular second messengers, however, the work of Linde and colleagues have vastly increased our knowledge regarding the role of odontoblasts in Ca^{2+} transport during dentinogenesis (Linde and Goldberg, 1993). Early reports pointed towards an enrichment of Ca^{2+} ions in predentin, compared to the levels present in rat tail tendon, suggesting a mechanism concentrating Ca^{2+} at the site of mineralization, and that Ca^{2+} ions were bound to some macromolecules (Nicholson *et al.*, 1977). Studies have established Ca^{2+} levels to be approximately three times higher in predentin compared to the levels in the pulp tissue, suggesting a Ca^{2+} ion concentrating mechanism across the cell layer towards the mineralization front (Lundgren *et al.*, 1992).

Transport of Ca^{2+} from the blood to the mineralization front has been proposed to occur mainly via a transcellular mechanism (Linde and Magnusson, 1975). A flux of Ca^{2+} is delivered into the mineral phase through a concerted action of Ca^{2+} uptake and extrusion mechanisms, through the cell plasma membrane (Linde, 1995), including Ca^{2+} activated-ATPase, derived from the ER (Linde and Magnusson, 1975; Lundgren and Linde, 1987), Na^{+}/Ca^{2+} exchanger (Lundgren and Linde, 1988) and L-type Ca^{2+} channels (Lundgren *et al.*, 1992). A simple model for Ca^{2+} flow across the odontoblast cell layer was recently proposed by Lundquist (Lundquist, 2002) (Figure 4). The hypothesis suggested Ca^{2+} to enter the cell through Ca^{2+} channels and diffuse across the cell, either in free form, bound to Ca^{2+} - binding proteins or sequestered in the ER, with a possible exchange of Ca^{2+} between the three routes. Finally, Ca^{2+} ions are extruded into the predentin by Na^{+}/Ca^{2+} exchangers (Lundquist, 2002).

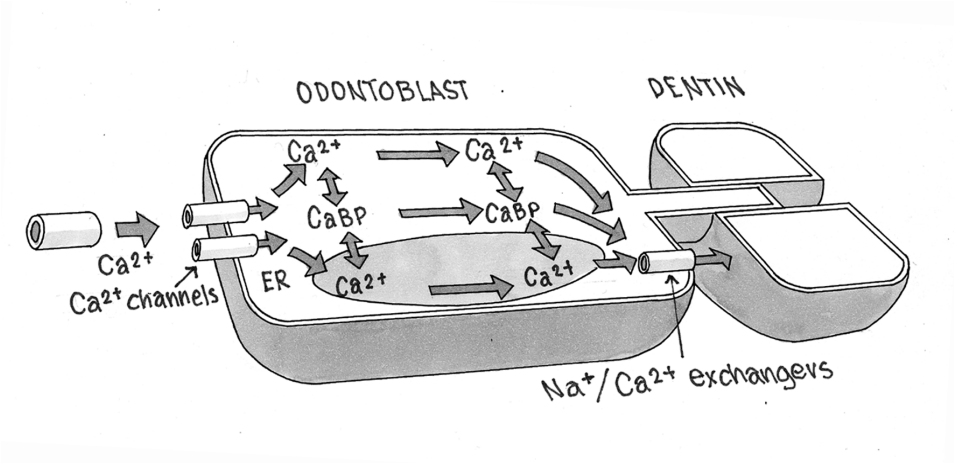

Figure 4. Schematical drawing of a proposed model for Ca^{2+} transport through the odontoblast. Ca^{2+} is believed to enter the cell through Ca^{2+} channels. It then diffuses across the cell bound to Ca^{2+} - binding proteins, in free form or sequestered in the ER. The Ca^{2+} ions are extruded out from the cell at the mineralization front.
CaBP=Ca^{2+} - binding protein, ER=endoplasmic reticulum

Ca^{2+} is a known regulator of a variety of cellular functions, including differentiation, proliferation, cellular motility, secretion, cytoplasmic and mitochondrial metabolism, protein synthesis, gene expression, cell-cycle progression, and apoptosis (Clapham, 1995; Pietrobon *et al.*, 1990; Pozzan *et al.*, 1994). Consequently, maintenance of a constant intracellular Ca^{2+} environment within the cell is necessary to ensure correct biological function. This stable milieu may be facilitated with, among other mechanisms, the presence of Ca^{2+} - binding proteins (Table 3). Ca^{2+}- binding proteins are proposed to associate with Ca^{2+} ions taken up by the cell and capable of complexing and storing large amounts of Ca^{2+} within cellular organelles. Due to the capacity of these proteins to bind and buffer free Ca^{2+} ions, intracellular Ca^{2+} levels can be maintained relatively constant, without any induction of unwanted Ca^{2+} - required biological mechanisms.

Table 3. **Ca^{2+} - binding proteins in bone and dentin**

Protein	Distribution and potential function
Calmodulin	Expressed in almost all eucaryotic cells Mediates many Ca^{2+} - regulated processes Plays a role during osteoclastogenesis
Calbindin	Expressed in a variety of cells Two molecular weight classes, calbindin D9k and calbindin D28k Regulates intracellular Ca^{2+} concentrations
Annexins	Expressed in a variety of cells, annexins III to VI are present in odontoblasts Implicated in initial mineralization within matrix vesicles
Calreticulin	Originally an ER luminal resident protein Expressed also extracellularly in mineralized tissues Chaperone and regulates intracellular Ca^{2+} levels Inhibits mineralization in osteoblastic cells
Nucleobindin	Expressed in the nucleus, Golgi and ER in a number of cells Expressed extracellularly Shares high sequence homology with calreticulin and NEFA Interacts with G-proteins Implicated in Ca^{2+}- regulated signal transduction events

Ca^{2+} - BINDING PROTEINS IN BONE AND DENTIN

Ca^{2+}- binding proteins are proposed to play a role in maintaining constant intracellular Ca^{2+} levels and the regulation of Ca^{2+} between different intracellular organelles. Several Ca^{2+} - binding proteins have been identified in bone and dentin and have been separated into different groups: the EF-hand containing proteins, the annexins, and within this thesis, calreticulin (CRT). The EF hand containing proteins calmodulin (Hubbard *et al.*, 1981; Lewinson and Boskey, 1984), calbindin (Berdal *et al.*, 1996; Taylor, 1984), parvalbumin (Celio *et al.*, 1984; Toury *et al.*, 1995) and nucleobindin (Nuc) (Wendel *et al.*, 1995) have been detected in bone and tooth.

Calmodulin, found in virtually all eucaryotic cells, plays a multifunctional role, mediating many Ca^{2+} - regulated processes. In dental tissues, calmodulin has been detected in rat incisor ameloblasts and odontoblasts during early stages of development (Goldberg *et al.*, 1987). Moreover, calmodulin has been identified in bone and cartilage in the growth plate area of developing long bones (Lewinson and Boskey, 1984). Calmodulin has also been shown to be present in high concentrations at the ruffled border of osteoclasts and suggested to play a role in osteoclastogenesis (Zhang *et al.*, 2003).

Calbindin is a soluble, vitamin D-dependent Ca^{2+} - binding protein. Two molecular weight classes of calbindins have been reported: a 8-10 kDa protein (calbindin D9k) and a 28 kDa protein (calbindin D28k). The presence of both molecules and their mRNAs have been established in cells of mineralized tissues (Berdal *et al.*, 1991; Berdal *et al.*, 1996; Onishi *et al.*, 1999). Calbindin D28k has been identified in ameloblasts (Berdal *et al.*, 1991), odontoblasts and osteoblasts (Berdal *et al.*, 1996). It has been speculated that the protein might play an important role in the initial mineralization process by regulating intracellular Ca^{2+} concentrations (Onishi *et al.*, 1999). Furthermore, mRNA for calbindin D9k has been detected in osteoblasts and ameloblasts, but has been reported to be very low or absent from odontoblasts (Berdal *et al.*, 1996).

Parvalbumin, also a vitamin D-dependent Ca^{2+} - binding protein, has been detected in developing rat teeth, specifically within the nucleus and cytosol of ameloblasts and to a lesser extent in odontoblasts (Davideau *et al.*, 1993). In bone, parvalbumin has been detected within the cytoplasm of osteoblasts, osteocytes and osteoclasts and also in chondrocytes of the epiphyseal cartilage, with the greatest intensity in the terminal hypertrophic chondrocytes of the calcifying zone (Toury *et al.*, 1995).

The annexins are a family of widely expressed Ca^{2+} and phospholipid-binding proteins, with a number of suggested functions in the regulation of membrane traffic, transmembrane channel activity, signal transduction events, apoptosis, blood coagulation processes, vesicle trafficking and Ca^{2+} signaling and transport. Initial mineralization has been proposed to occur through cell-derived, membrane-bound matrix vesicles. These contain several specific proteins, including alkaline phosphatase and annexins II, V, and VI. As a possible initial step for the formation of the first mineral phase within the vesicle lumen, these annexins are thought to form Ca^{2+} channels enabling Ca^{2+} influx into the vesicle (Kirsch *et al.*, 1997). Annexins I-VI have been detected in secretory ameloblasts of rat incisor, although only annexins III - VI were present in odontoblasts (Goldberg *et al.*, 1990).

Nucleobindin (Nuc)

Nuc, an EF hand containing Ca^{2+} - binding protein, first isolated from a B-lymphocyte cell line, established from mice prone to develop the autoimmune disorder systemic lupus erythematosis (Kanai *et al.*, 1986). Nuc structure is composed of at least four functional motifs, including a signal peptide, DNA-binding site, two EF-hand motifs and a leucine zipper (Miura *et al.*, 1992) (Figure 5). Interestingly, sequence alignments show Nuc to exhibit a significant homology (60%) with CRT. Furthermore, the protein NEFA (DNA binding, EF-hand, Acidic region) another EF hand family member, has been shown to share high sequence identity (62%) to Nuc (Karabinos *et al.*, 1996). The human gene for Nuc has been localized to chromosome 19 and is composed of 13 exons that span a region of 32 kb, with each functional motif encoded by a separate exon (Miura *et al.*, 1996).

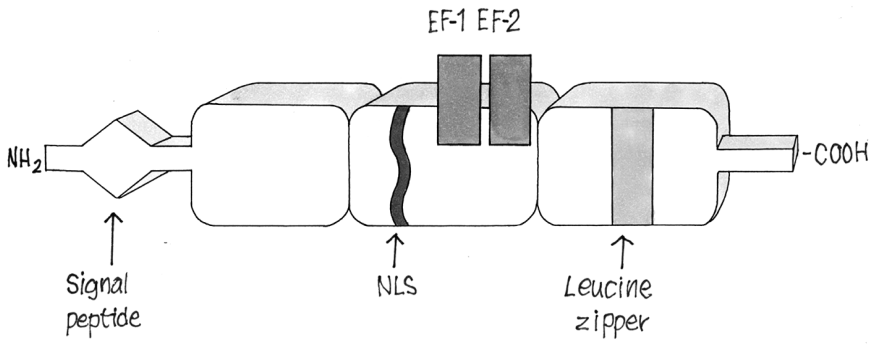

Figure 5. Structural features of Nuc, showing an N-terminal signal sequence, a putative DNA-binding domain with a nuclear localization signal (NLS), an acidic region flanked by two Ca^{2+} - binding EF-hand motifs (EF-1, EF-2) and a leucine zipper motif.

Considering the ability of Nuc to bind to DNA fragments *in vitro*, the protein was initially thought to be a transcription factor and termed Nuc (Miura *et al.*, 1992). Later, Wendel et al (Wendel *et al.*, 1995) isolated and determined the primary structure of the protein from bovine bone and demonstrated that Nuc was a biosynthetic product of newborn rat calvarial explants in culture. Nuc has also been shown to be ubiquitously distributed, and has been described as a nuclear protein (Miura *et al.*, 1994), (Wang *et al.*, 1994), a secreted protein (Miura *et al.*, 1992), (Wendel *et al.*, 1995), and as a Golgi resident protein (Lin *et al.*, 1998). Nuc has been detected in the lumen of the ER, where it interacts with cyclooxygenases (Ballif *et al.*, 1996). The secretion pathway for Nuc has been investigated, and it was shown that in AtT20 cells, a human pituitary cell line known for well characterized secretory pathways, Nuc was synthesized in the ER, transported to the Golgi, where it was post-transcriptionally modified (O-glycosylated and sulfated), and subsequently released after some time period from the cell via constitutive-like secretory pathways (Lavoie *et al.*, 2002).

A variety of functions have been described for Nuc, including Ca^{2+} - binding in the Golgi (Lin *et al.*, 1999), interacting with G-proteins and it has also been postulated to posses an important role in Ca^{2+} - regulated signal transducing events (Lin *et al.*, 1998). Results provided by Lin et al., 1999 showed that Nuc bound to Ca^{2+} *in vivo* and together with the Ca^{2+} pump, sarcoplasmic/ER Ca^{2+} ATPase (SERCA) and IP_3 receptor type I (IP_3R-1) is involved in the maintenance of Ca^{2+} storage in the Golgi (Lin *et al.*, 1999). Moreover, functional investigations using recombinant Nuc, demonstrated that it was the first of the two EF hands, which were involved in Ca^{2+}-binding (Miura *et al.*, 1994).

The importance and potential functions of Nuc will be discussed later within this thesis.

Calreticulin (CRT)

CRT, an ER luminal resident protein has two major functions; chaperoning and regulating intracellular Ca^{2+} levels. CRT was isolated in 1974, and was originally identified as a sarcoplasmic/endoplasmic membrane protein (Ostwald and MacLennan, 1974). The protein was also localized to the bloodstream (Sueyoshi *et al.*, 1991), cell surface (Arosa *et al.*, 1999; Basu and Srivastava, 1999) and nucleus (Holaska *et al.*, 2001). CRT has been shown to be an ancient and highly conserved protein, found in a wide range of species (Coppolino and Dedhar, 1998). CRT comprises three distinct domains; the N-domain, P-domain and C-domain (Fliegel *et al.*, 1989; Michalak *et al.*, 1992; Smith and Koch, 1989) with different functions (Figure 6). The P- and C-domains have high affinity and high capacity Ca^{2+} - binding sites respectively, the C-domain seems to determine the Ca^{2+} storage capacity of the ER, along with the participation of the P-domain (Nakamura *et al.*, 2001).

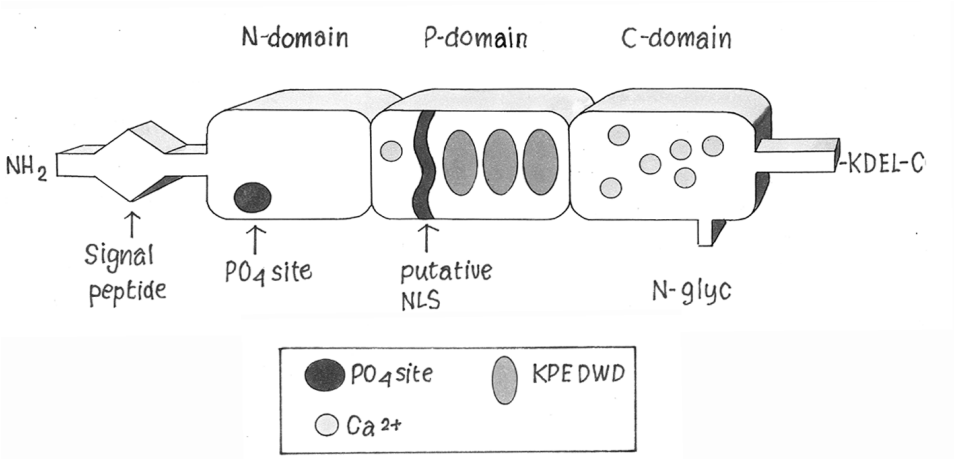

Figure 6. Schematical drawing of CRT, showing the three functional domains. Located at the N-domain is a putative phosphorylation site and a segment which binds to steroid hormone receptors. The P-domain contains a high-affinity, low capacity Ca^{2+}-binding site, a putative nuclear localization signal and several KPEDWD repeats. The C-terminal third contains the low-affinity/high capacity Ca^{2+}-binding site, a potential site for N-linked glycosylation and a KDEL endoplasmic reticulum retrieval sequence.

Due to the Ca^{2+} - binding function, CRT has been suggested to be of importance during the mineralization process and subsequently, the involvement of CRT has been studied in enamel development (Hubbard, 1996). CRT was reported to be highly upregulated when dental enamel became fully mineralized, with the ameloblasts requiring Ca^{2+}-binding proteins to regulate the large amounts of Ca^{2+} ions present (Hubbard, 1996). Interestingly, CRT has been shown to play an important regulatory role in osteoblast function, differentiation and bone formation, where the constitutive expression of CRT inhibited mineralization in the osteoblastic cell line MC3T3-E1, a well-characterized mouse osteosarcoma cell line (St-Arnaud *et al.*, 1995). This regulation of mineralization was shown to occur by CRT protein – protein binding to the vitamin D receptor and thereby regulate osteoblastic differentiation and function (St-Arnaud *et al.*, 1995). Potential functions of CRT during the mineralization process of dentin will be discussed later within this thesis.

BIOMINERALIZATION

The process of biomineralization involves the deposition of an inorganic mineral phase within an organic ECM framework (Boskey, 1998). The osteoblasts or odontoblasts direct the mineral formation process by expression of proteins that either act as nucleators in the extracellular matrix or prevent mineral formation in unwanted sites, by production of enzymes that modify the functions of these proteins, and by the regulation of ion transport (Boskey, 2003). Two types of structures appear to be relevant in promoting mineralization; lipid membranes that provide protected environments in which initial mineral forms, and proteins that serve as nucleators and regulators of crystal growth and orientation, such as DPP, BSP and biglycan (Boskey, 2003). The properties of the mineral in bone and dentin have been reported to be somewhat dissimilar. Dentin mineral crystals have been described as being larger and more perfect than bone mineral crystals following X-ray diffraction and infrared imaging (Arnold *et al.*, 2001). The proteins involved in bone and dentin mineralization are similar, but distinct. In comparison to bone, dentin has been shown to contain less OPN, BSP and osteocalcin, but more DPP and DMP-1. Odontoblasts are more polarized than osteoblasts, and the collagen fibrils in predentin and newly formed dentin have been reported to be thicker and more orientated than in osteoid and newly formed bone (Linde and Goldberg, 1993).

Two processes have been proposed to be involved in mineral crystal formation in hard tissues. The first, termed homologous nucleation, has been shown to take place when a solution is supersaturated regarding the forming salt, and may include membrane bound matrix vesicles (Anderson, 1969). The cell derived matrix vesicles provide a protective microenvironment for crystallization and are believed to play a role during initial mantle dentin formation in teeth, although the mechanisms for initial mineral formation remains to be totally clarified (Linde and Goldberg, 1993). The vesicles contain different nucleating molecules, such as SLRPs, Ca^{2+}-binding proteins (calmodulin and annexin V),

phospholipids and various enzymes including alkaline phosphatase, pyrophosphatase, Ca-ATP-ase and metalloproteinases (Linde and Goldberg, 1993). The second nucleating process, heterogenous nucleation or matrix-mediated mineralization, involves the formation of crystals guided by an unmineralized ECM that acts as a catalyst to crystal formation (Linde and Goldberg, 1993). Crystals are deposited in relation to collagen fibrils, although it should be noted that collagen itself does not probably have any nucleating function. Instead, several of the ECM proteins secreted by the cells, possess the potential to act as initiators of crystal growth (Glimcher, 1989). It is generally acknowledged that the initial mineral appears in the gap zones at the ends of collagen fibrils (Lees and Prostak, 1988), where the Ca^{2+} - binding SLRPs are located. Ca^{2+} remains within the gap zones when the SLRPs are enzymatically modified by MMPs, leaving the core protein associated with collagen fibril, and the phosphoproteins are now able to bind to the collagens. Dephosphorylation of these proteins allows additional phosphate ions in the region, permitting progression of crystal growth (Embery *et al.*, 2001).

Embery and co-workers have proposed a model for the mechanism of calcification, based on the dentinogenesis process, suggesting a prominent role for SLRPs in directing the deposition of mineral crystals at the mineralization front (Embery *et al.*, 2001). Two pools of PGs have been identified during dentinogenesis; one in predentin, which appeared to be secreted together with collagen fibers and which is rapidly metabolized, and a second, more stable, mineral-bound pool, secreted close to the predentin-dentin interface (Linde *et al.*, 1980). Embery et al., suggested a main role for the predentin pool of PGs in type I collagen organization towards the mineralization front (Embery *et al.*, 2001). Moreover, a gradient of the various PGs present was suggested to be of significance during the mineralization process. CS immunoreactivity has been shown to decrease towards the mineralization front (Septier *et al.*, 1998), and this is in contrast to the response of an anti-decorin antibody, which was found to increase closer to the predentin-dentin interface (Septier *et al.*, 2001). Thereby, inferring that the lack of GAG witnessed towards the predentin-dentin interface was due to modification of the PG by various MMPs (Hall *et al.*, 1999), leaving the core protein sequestered in the collagen gap zone. The group suggested that phosphoproteins and phospholipids associate closely with the remaining core protein, by unknown mechanisms, and initiate mineral deposition with precipitation of Ca^{2+} P_i. Interestingly, an alteration in GAGs moieties toward the mineralization front has also been reported, showing, as mentioned above, a decrease in CS, but an increase in KS close to the mineralization front, indicating a potential role for KS-substituted SLRPs in initiating HAP deposition (Goldberg *et al.*, 2003).

AIM OF THE PRESENT INVESTIGATIONS

The overall aim of the project was to study the protein composition in dentin and identify new components and their potential functions. Dentinogenesis is considered a good model to investigate the complex events involved during biomineralization and it possesses several important advantages in comparison to the study of osteogenesis. The morphology of the different components (cells/predentin/dentin) is distinct and well separated, dentin does not participate in calcium homeostasis of the body and it does not remodel, except from shedding.

Three matrix components have been extensively investigated; OSAD, Nuc and CRT, which all appear to play a crucial role in the mineralization process of bone and dentin.

Specific aims

-To investigate if OSAD, a bone proteoglycan, was present within the tooth, and if so, study the localization of OSAD in developing dental tissues.

-To investigate if Nuc, an earlier discovered Ca^{2+} - binding protein present extracellularly in bone, was present in tooth and if so, to study the localization of Nuc within dental tissues.

-To further elucidate localization of Nuc in bone tissue and investigate the role of Nuc during the mineralization process of primary osteoblasts grown under osteogenic conditions.

-To investigate the localization of CRT within dental tissues. CRT was for the first time discovered to be present in dentin and for the first time revealed to be secreted to the extracellular matrix.

-To establish an *in vitro* long bone organ culture system in order to investigate the potential effects of the detailed NCPs during endochondral bone formation by knocking down the specific gene.

MATERIAL AND METHODS

Table 4. **Modus operandi**

Manuscript	*Overview of techniques used*
I	**Biochemical characterization of OSAD**: protein extraction, protein separation on ion-exchange chromatography column, SDS-PAGE, protein identification by Western blot analysis and N-terminal sequencing **Morphological distribution of OSAD**: immunohistochemistry, immuno-electron microscopy **Identification of OSAD gene expression**: Northern blot analysis, *in situ* hybridization
II	**Characterization of Nuc in tooth**: antibody preparation, SDS-PAGE, Western blot analysis, immunohistochemistry, immuno-electron microscopy **Identification of Nuc gene expression in tooth:** Northern blot analysis, *in situ* hybridization
III	**Characterization of Nuc in bone**: Immunohistochemistry, immuno-electron microscopy **Identification of Nuc gene expression in bone**: Northern blot analysis, i*n situ* hybridization **Role of Nuc during the mineralization process**: calvaria cell culture in osteogenic conditions, Northern blot analysis, RT-PCR, immunohistochemistry, von Kossa staining
IV	**Biochemical characterization of CRT**: protein extraction, protein separation on ion-exchange chromatography and HAP columns, SDS-PAGE, protein identification by Western blot analysis and N-terminal sequencing **Morphological distribution of CRT**: immunohistochemistry, immuno-electron microscopy **Identification of CRT gene expression**: Northern blot analysis, *in situ* hybridization **Regulation of CRT**: culture of rat dental pulp cells and vitamin D treatment
V	**Role of BSP in endochondral bone formation**: designing antisense oligos, MG3T3-E1 cell culture, metatarsal bone organ culture, RT-PCR, immunohistochemistry

PRESENT INVESTIGATIONS AND RESULTS

A mineralized tissue such as bone or dentin comprises a large inorganic phase, consisting of a calcium phosphate salt named HAP, and a smaller organic part, composed of collagens and NCPs. The collagens provide the tissue with structural support and the NCPs are believed to possess important roles in organizing the collagen matrix and in regulating the formation and growth of the HAP crystals. Regardless of the fact that these proteins have been extensively studied, a full comprehension of the function of the various molecules has not been established. One option to increase the knowledge concerning the function of a particular protein within the bone or dentin matrix would be to identify a specific localization and/or co-localization with other known matrix molecules in the tissue. Within this thesis, the localization of three different extracellular matrix proteins, OSAD, Nuc and CRT, have been studied and it appears that they participate and play a crucial role in the mineralization process of bone and dentin. Furthermore, an *in vitro* long bone organ culture system was set up, using antisense oligonucleotides to knock down gene expression of BSP, in order to elucidate the role of BSP in endochondral bone formation.

Expression of OSAD in developing tooth (paper I)

OSAD has previously been biochemically characterized from bovine long bone and immunolocalized in the primary spongiosa of fetal rib growth plate of bovine bone. Furthermore, the gene expression of OSAD has been detected in osteoblasts lining the surface of bone trabeculae, especially strong at the interface between cartilage and bone (Wendel *et al.*, 1998). Since bone and dentin possess some similarities concerning biochemical composition and molecular constituents, the hypothesis in the first paper was that OSAD might be present in dentin as well.

To examine this theory, bovine teeth were collected and dentin of incisors from young cows was used. The dentin was ground into powder after free dissection from enamel, cementum and pulp, and the mineral associated proteins were extracted in sequences using a procedure first introduced by Termine et al., (Termine *et al.*, 1981a), and further developed by Franzén and Heinegård (Franzen and Heinegard, 1984). In the first step, the frozen dentin powder was extracted in 4 M guanidine-HCl to exclude the proteins, which are not bound to the mineral phase. The remaining proteins were further extracted in 4 M guanidine-HCl containing EDTA, which dissolved the crystals and released the mineral bound proteins. The decalcified dentin protein extract was then concentrated and transferred into 7 M urea, to allow separation of the proteins with a variety of chromatographic steps. A similar bovine bone extract had already been produced and was available for us to use.

OSAD was partially purified from the dentin extract on a DEAE-cellulose (DE52) anion-exchange column, which was eluted with a linear sodium acetate gradient. Fractions were collected and run on SDS-PAGE and in fraction 14, a

protein band at approximately 85 kDa was detected, with a similar elution profile as for bone OSAD. The fraction was transferred to a nitrocellulose membrane and the 85 kDa band was excised. The OSAD identity was confirmed by N-terminal sequencing and was further identified by Western blot analysis of the total dentin extract with an affinity purified antibody against bovine OSAD, produced in rabbit. Western blot analysis, including proteins from both bovine bone and dentin, revealed two bands in the two tissues at 85 kDa and 60 kDa. In bone, the band at 85 kDa was prominent and the second band at approximately 60 kDa was weaker, although in the dentin protein extract an opposite expression pattern was apparent, with the 60 kDa band being more abundant than the 85 kDa band. The 85 kDa band probably corresponded to the fully glycosylated PG and the 60 kDa band to the core protein absent of KS chains, suggesting that most of OSAD present in dentin contains no KS. This result was in agreement with Wendel et al., (Wendel *et al.,* 1998), who showed that by treating the 85 kDa PG with keratanase, the molecules mobility increased on SDS-PAGE to 60 kDa, thereby suggesting the presence of KS chains. Treatment of OSAD, metabolically labelled with [^{35}S]sulfate, with N-glycosidase F demonstrated that mobility on SDS-PAGE was increased from 85 kDa to 47 kDa, thus also indicating that OSAD may contain other sulfate containing groups, such as tyrosine sulfate (Wendel *et al.*, 1998).

To further characterize and identify OSAD in dental tissues, Northern blot analysis of RNA samples from 5-day-old rat tooth and femur was performed, which showed a 1.9 kb transcript for OSAD in both tissues. In addition, paraffin sections from a 2-day-old rat maxilla were hybridized with a ^{35}S-UTP-labeled RNA probe for OSAD. During early crown stage, OSAD was highly expressed in the entire odontoblast layer where mineral deposition was barely apparent or had not yet been initiated. At a later stage, OSAD was distinctly expressed in the area of Hertwig´s epithelial root sheath, restricted only to the cells of mesenchymal origin. OSAD was intensely expressed in the cells adjacent to the newly formed mantle dentin, but expression appeared to decrease with the progression of mineralization. Furthermore, expression of OSAD was also detected in the cells of developing alveolar bone, which surrounded the teeth.

The affinity purified antibody against OSAD was used to perform immunohistochemistry on paraffin sections from bovine incisors, which were decalcified for three months. Within dental tissues, OSAD was localized to the dentin matrix and cementum, although the most intense labeling of OSAD was detected in the surrounding alveolar bone. The bovine antibody was also tested on rat sections, but no cross-reaction seemed to occur. A peptide antiserum against two different peptide sequences (one at the very N-terminal of OSAD, amino acids 28-40, and the other more C-terminally, amino acids 360-374) of rat OSAD was produced in rabbit and tested by immunohistochemistry on rat sections. Both peptide antisera showed good labeling in rat bone, which was in full agreement with earlier reported localization of OSAD (Shen *et al.*, 1999). No positive staining for OSAD could be detected within dental tissues, which was surprising in considering the clear gene expression of OSAD. Recently however, a peptide

antiserum against the N-terminal of rat OSAD has been produced in egg yolk, which shows weak labeling not only in bone, but also in dentin and odontoblasts.

Ultrastructural studies of rat dental tissues detected OSAD within the ER of young odontoblasts, further confirming synthesis of the protein by these cells. Extracellularly, OSAD labeling was found to be in association with collagen fibers.

Taken together, these results suggest that OSAD possesses important functions during tooth development and biomineralization of dentin. Potential roles for OSAD, in consideration of related PGs localization and known functions, and the capacity of OSAD to bind HAP, could be to act as a regulator of HAP nucleation, possibly as a nucleator. Moreover, due to OSADs close association with type I collagen fibres, another potential function could be to bind and regulate fibrillogenesis. It has also been reported that OSAD appears to be upregulated by TGFβ (Lucchini *et al.*, 2002) and if also involved in collagen fibrillogenesis, OSAD could play a role in organizing the collagenous matrix and its biomineralization.

Nuc in dental and bone tissues (paper II and III)

Nuc has previously been identified as a secreted protein in bone ECM and the primary structure was determined from bovine bone (Wendel *et al.*, 1995). To further characterize and localize Nuc within bone and tooth tissues, a peptide antibody was generated in rabbit against the C-terminal of the Nuc sequence, amino acids 439-455. The sequence chosen for peptide antiserum production was due to Nuc's high sequence homology to another protein, termed NEFA, in order to exclude cross-reaction of the antibody. The antibody recognized a band at approximately 63 kDa of rat bone and dentin total extracts, by Western blot analysis, which agrees with previous studies (Wendel *et al.*, 1995). Within the tooth tissues, Nuc was immunolocalized to the mature odontoblast layer, the dentin and also in secretory ameloblasts. Positive staining for Nuc was also observed within the osteoblasts of the surrounding bone. In developing rat femur bone, Nuc was detected in osteoblasts lining the newly mineralized bone trabeculae, in osteocytes and in hypertrophic chondrocytes in the area of the growth plate. Additionally, osteoblasts and osteocytes localized in rat calvaria showed immunostaining for Nuc.

In dental tissues, immuno-electron microscopy detected Nuc in mature odontoblasts within the ER, mitochondria, cytoplasm and in the nucleus. Furthermore Nuc was detected extracellularly in the predentin and dentin matrix. In bone tissue, the most intense immunolabeling was detected extracellularly in the osteoid of newly formed bone and intracellularly within the rough ER and nucleus of osteoblasts. Moreover, staining was detected in the cytoplasm of osteoclasts, in chondrocytes and in newly mineralized septae between columns of hypertrophic chondrocytes. The ultrastructural distribution of Nuc within the growth plate of rat tibia was compared to the localization of two widely studied bone proteins, BSP and OPN. The three proteins were all identified in the area, although the distribution varied slightly. Nuc showed a narrower and more distinct localization closer to the cells compared to both BSP and OPN.

In order to study the synthesis of Nuc in bone and dentin producing cells, in *situ* hybridization was performed on paraffin sections, using both radioactively labeled (^{35}S) RNA probes and non-radioactive probes (DIG-labeled). Within the tooth, a specific signal for Nuc mRNA was detected in odontoblasts by the ^{35}S labeled antisense probe, and the expression appeared to increase as the odontoblasts matured. In developing bone, Nuc expression was localized, with a DIG-labeled antisense probe, within the osteoblasts and osteocytes of newly formed bone close to the growth plate of rat femur, and in calvarial bone, Nuc mRNA was detected within osteoblasts and osteocytes with the ^{35}S-labeled probe. These results clearly show that Nuc is synthesized by the various bone cells and by the odontoblasts.

To further elucidate the role of Nuc during the mineralization process, rat calvaria-derived primary osteoblasts were grown under osteogenic conditions. Total RNA was isolated at different time points, approximately corresponding to cell proliferation, differentiation, nodule formation and mineralization. RT-PCR and Northern blot analysis was performed on RNA from the various time points. By RT-PCR, Nuc expression was shown to be low during cell proliferation (day 4), and upregulated with differentiation and commencement of nodule formation (days 7-14). The levels of Nuc were subsequently downregulated with the onset of mineralization (days 21-28). This data agreed with the Northern blot analyses, where Nuc expression was low during proliferation of osteoblasts and appeared to increase with differentiation and nodule formation (days 7-14). After mineralization had started, Nuc expression seemed to diminish (days 21-28). Furthermore, the osteoblastic cells were grown on chamber slides and immunostained with the peptide antibody on the indicated time points. Positive staining of Nuc was localized strictly within the nucleus of the cell during cell proliferation and differentiation (day 4 – 7), while after the onset of mineralization (day 28) a shift in intracellular expression occurred. Positive labeling of Nuc was at this time point detected in the cytoplasm, with only low levels in the nucleus of the cells. Moreover, staining for Nuc was localized preferentially in the cells forming the mineralized nodules.

These data suggest Nuc to be synthesized by osteoblasts and odontoblasts and that Nuc might play a role during biomineralization of bone and dentin. A possible role for Nuc in dental tissues is to bind and transport Ca^{2+} ions from the circulation, through the cell, and out into the mineralization front prior to HAP deposition. Furthermore, the co-localization of Nuc with BSP and OPN, both of which have been described to have HAP regulatory roles during tissue mineralization, might suggest that Nuc could play a regulatory role during bone and dentin mineralization. Concerning Nuc's known Ca^{2+} - binding function, and that Nuc has been shown to be localized both extracellularly and within the nucleus, it could also be speculated that Nuc might act as a Ca^{2+} - sensing protein in the extracellular matrix, possessing functions as a shuttle molecule. Nuc might be transported back into the nucleus, where it could act as a transcription factor, thereby influencing the mineralization process.

CRT in ECM of dental tissues (paper IV)

In the search for novel dentin matrix proteins, several fractions from the DEAE-column, previously described in the OSAD paper, were visualized on SDS-PAGE and selected for further analyses. Within fractions 17 and 18 from the anion-exchange chromatographic step, a protein band at approximately 59 kDa on the SDS-PAGE was of interest. The pooled proteins from these two fractions were further separated on a HAP column, and SDS-PAGE of the eluted fractions revealed a major band at 59 kDa in fractions 31 and 32. This protein band was excised, N-terminally sequenced and was subsequently identified to be CRT. Interestingly, CRT has been described as a Ca^{2+} - binding ER luminal resident protein, also detected in the nucleus, at the cell surface and in the bloodstream. However until now, the protein has not been reported to be present extracellularly. The identity was further confirmed by sequencing of internal peptides and by Western blot analysis of total rat bone and dentin protein extracts, using an affinity purified peptide antibody, mapping at the amino terminus of CRT and produced in goat. Western blotting revealed two distinct bands in bone and dentin, one the full-length molecule at 59 kDa, the other at 42 kDa. The smaller band could possibly correspond to a shorter version of CRT or to a potential degradation product, which has earlier been reported to occur.

In order to localize CRT within dental tissues, immunohistochemistry was performed on paraffin sections of 5-day-old rat heads, using the goat produced peptide antibody. CRT was localized to the odontoblasts and ameloblasts. The temporo-spatial expression pattern of CRT revealed a gradual increase of immunoreactivity along with maturation of the cells, showing low staining in preodontoblasts, followed by an upregulation in secretory odontoblasts. In addition, the ameloblasts showed the same expression pattern, with increasing signal with maturation of the cell. A weak signal was observed in the peritubular dentin around the odontoblast processes and CRT staining was also present in the osteoblasts of the surrounding alveolar bone. By ultrastructural immunohistochemistry, the detected CRT staining was slightly contradictory to the results obtained by normal immunohistochemistry. The cell labeling was high in early odontoblasts, with a subsequent decline in more mature cells. Within the odontoblasts, CRT was localized in the nucleus, ER and low levels in the cytoplasm. The staining in the ER followed the pattern of cell labeling, with decreasing levels in more mature cells. However, within the predentin matrix, the labeling of CRT, increased towards the coronal part of the tooth.

In order to study the effects of vitamin D on the amount of exported CRT, rat pulp cells were treated with vitamin D. An upregulation of CRT levels were detected in the cell medium, compared to untreated medium, suggesting an increased synthesis of CRT by these cells as a result of the vitamin D supplementation. In addition, CRT was detected in the ECM of the rat pulp cells, further confirming that CRT was present extracellularly.

To further investigate the synthesis of CRT in dental tissues, *in situ* hybridization was performed. Consistent with the ultrastructural data, mRNA for

CRT was detected at high levels in young odontoblasts, with a downregulation in expression with maturation.

Taken together, these data suggest that CRT plays a role during mineralization of dentin. Previously it has been shown that CRT is upregulated during enamel development (Hubbard, 1996). It was shown that during the enamel mineralization process, the ameloblasts required Ca^{2+} - binding proteins in order to regulate the large amounts of intracellular Ca^{2+} present, and consequently, CRT was upregulated. Our results clearly show that CRT is synthesized by odontoblasts and subsequently secreted out from the cell. During dentin mineralization CRT may participate in an intracellular Ca^{2+} transport route within the odontoblasts and act as a Ca^{2+} carrier to the mineralization front. Interestingly, CRT showed a similar expression pattern to Nuc within dental tissues, suggesting that CRT might work in concert with Nuc in binding and transporting Ca^{2+} ions to the mineralization front prior to HAP deposition.

BSP in bone formation (paper V)

In order to study the potential function of a specific protein, an *in vitro* long bone organ culture system has been set up. Antisense technology was used to knock down the BSP gene and investigate the effects on long bone formation. BSP has been shown to mediate cell attachment and has been described to act as an initiator of HAP nucleation. However, BSP knockout mice have not showed any definite bone phenotype, indicating possible compensatory functions by other proteins with similar roles.

Metatarsal bones from embryonic mice (embryonic day 16) and an osteoblastic cell line, MC3T3-E1, were cultured with or without the presence of BSP antisense oligonucleotides (oligos), which were labeled with FITC to be able to track the oligo within the cells or organ by fluorescence microscopy, ensuring cellular uptake and distribution of the oligos. The oligos were delivered into the cells or organs either by electroporation or by passive diffusion of oligos added to the cell culture medium.

It was clearly shown that the *in vitro* organ culture system was a reproducible and illustrative method to study the mineralization process in long bones. With few exceptions, all bones started to mineralize at day two in culture and, when kept in culture for up to 12 days, appeared to develop a normal growth plate in comparison to in vivo situations, although the process was much slower. Moreover, the oligos undoubtedly penetrated the cells and tissues, detected by fluorescence microscopy, and there was no noticeable difference in cellular uptake between passive diffusion and electroporation. Total RNA was isolated from cells and metatarsals, and RT-PCR was performed in order to elucidate knock down efficacy of the oligos. No knock down effect of the BSP gene was detected, either in RNA from cells or metatarsal, and additionally, no morphological effect on the rate of growth and rate and degree of mineralization was visible, indicating non-function of the chosen antisense oligos. Immunohistochemistry was also performed on frozen sections of the metatarsals, cultured for one and three days, with an

affinity purified antibody against mouse BSP. Within the metatarsals, BSP was immunolocalized to osteoblasts, osteocytes, chondrocytes and in the bone matrix of cultured metatarsals at day one and day three. However, BSP was also detected in explants cultured without adding antisense oligos, and the positive staining for BSP showed equally levels of intensity, again indicating non-function of the oligos.

Taken together, the lack of influence on mineralization by knocking down the BSP gene does most likely not depend on lack of effects of BSP within this process, but rather on the oligos chosen. Therefore, further sequences need to be tested in order to elucidate the role of BSP on the mineralization process.

CONCLUSIONS

Within this thesis the main purpose was to search for novel dentin matrix proteins and eventually elucidate potential functions within dental tissues. The starting material was bovine root dentin, from which specific proteins were extracted and characterized. Furthermore, morphological, cellular and molecular biological techniques were used, in order to establish tissue localization of the proteins and their genes. In parallel, the specific localization and potential function of the proteins were studied in bone tissue as well. To facilitate functional studies, an *in vitro* long bone organ culture system was set up, using the antisense technique to knock down the gene expression of specific molecules.

The following conclusions can be drawn from the herein presented investigations:

Paper I: Identification, distribution and expression of osteoadherin during tooth formation, Petersson U, Hultenby K and Wendel M (2003), *Eur J Oral Sci*, Apr;111(2):128-36

- OSAD is present in dental tissues within mineralized dentin and cementum.
- OSAD appears to be associated with type I collagen fibrils.
- OSAD appears to be present in dentin in a less glycosylated mode compared to bone.
- Transcripts of OSAD during early dentinogenesis are localized in mesenchymal cells in the area of Hertwig´s epithelial root sheath and in the odontoblasts adjacent to the newly formed mantle dentin, with a decrease in expression during progression of mineralization.
- OSAD is synthesized by odontoblasts.
- Potential functions of OSAD within the tooth are to regulate dentin biomineralization, regulate type I collagen fibrillogenesis and to interact with TGFβ-1.

Paper II: Nucleobindin - a Ca^{2+}- binding protein present in the cells and mineralized tissues of the tooth, Somogyi E*, Petersson U*, Sugars RV, Hultenby K and Wendel M, accepted in *Calcif Tissue Int*, * Authors have contributed equally to this study

Paper III: Nucleobindin is a potential regulator of mineralization in bone cells and extracellular matrix, Petersson U, Somogyi E, Reinholt FP, Klinge B, Sugars RV and Wendel M, submitted to *Bone*

- Nuc is present in dental tissues within the dentin matrix, odontoblasts and ameloblasts.
- Nuc is also present within bone matrix in the osteoid of newly formed bone and in compact bone.

- Nuc is synthesized by odontoblasts and by all the various bone cells.
- Nuc is present in cartilage and chondrocytes, although to a lesser extent than that found in bone.
- In osteoblastic cells Nuc is expressed in low levels during proliferation and is upregulated during differentiation and nodule formation, followed by a downregulation after the onset of mineralization.
- Potential functions of Nuc during biomineralization of bone and dentin are binding and transporting Ca^{2+} ions to the mineralization front prior to HAP deposition, regulating HAP nucleation and acting as a Ca^{2+} - sensing protein in the ECM.

Paper IV: Calreticulin - an endoplasmic reticulum protein with calcium-binding activity is also found in the extracellular matrix, Somogyi E, Petersson U, Hultenby K and Wendel M (2003), *Matrix Biol*, Apr;22(2):179-91

- CRT was for the first time identified as an extracellular protein.
- In dental tissues CRT was identified in odontoblasts, ameloblasts and in predentin.
- CRT is synthesized by and secreted from odontoblasts.
- CRT secreted from rat pulp cells was upregulated following vitamin D supplementation.
- Suggested functions of CRT in dental tissues are to participate in an intracellular Ca^{2+} transport route within the odontoblasts and act as a carrier of Ca^{2+} ions from the circulation to the mineralization front.

Paper V: The role of bone sialoprotein in endochondral bone formation, Petersson U, Sugars RV, Ganss B, Klinge B and Wendel M

- The metatarsal *in vitro* organ culture system is a good model to study endochondral bone formation.
- Antisense oligos penetrate cells and tissues and there appears to be no detectable difference in cellular uptake between passive diffusion and electroporation.
- Various antisense oligos possess different efficacy to knock down gene expression and therefore, several sequences need to be evaluated.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Biomineralization is a dynamic process where the NCPs are believed to play a major role in the ECM organization and the regulation of nucleation. Within this thesis, three ECM proteins; OSAD, Nuc and CRT have been localized and characterized in bone and dental tissues. From their specific localization all the proteins could be inferred to be of importance during the mineralization process.

Several studies have suggested important regulatory roles for the SLRPs during dentin mineralization (Embery *et al.*, 2001; Hall *et al.*, 1997; Sugars *et al.*, 2003). The current investigation has focused on the class II SLRP, OSAD, substituted with KS chains, and found it to be present in dentin. The function of OSAD in the mineralization process remains to be fully elucidated. However, taking into consideration the localization of OSAD observed in this study, and the ability to bind HAP, it is tempting to speculate on the role of this molecule in biomineralization. The model of dentinogenesis, proposed by Embery and colleagues (Embery *et al.*, 2001) provides an important outline of the functions and interactions of other members of the SLRP super-family in the mineralization process. The model was based on earlier reports of a gradient distribution of class I and II SLRPs and their GAG moieties, across the predentin towards the mineralization front, and previously described functions of SLRPs, including the binding to type I collagen, Ca^{2+} and HAP (Septier *et al.*, 1998; Sugars *et al.*, 2003). Although, it should be noted that information concerning functions of class II SLRPs in the mineralization process is limited, in comparison to the studies performed on the class I SLRPs, decorin and biglycan. Lumican, another KS-substituted SLRP, has demonstrated immunohistochemical localization to increase towards the mineralization front, coinciding with an increasing gradient of KS epitopes (Hall *et al.*, 1999). However, within our study, OSAD was only detected within the mineralized dentin, which therefore suggests a potential different role for OSAD in the mineralization process. Lumican is also known to regulate collagen fibrillogenesis, by binding to type I collagen fibrils (Svensson *et al.*, 2000). OSAD, on the other hand, was found to be in close association with type I collagen fibrils, although, full investigation as to whether OSAD binds to type I collagen and influences collagen fibrillogenesis needs to be determined.

SLRPs have been described to be multi-functional in the mineralization process, acting as regulators, inhibitors and nucleators of mineralization. Decorin and biglycan have been reported to possess the ability to bind HAP and regulate crystal growth (Sugars et al., 2003). Indeed, OSAD has also been described to bind HAP (Wendel et al., 1998) and a recent study has described the co-localization of OSAD and BSP in bone (Ramstad et al., 2003). BSP is known to promote the nucleation of HAP crystals *in vitro* and bind to the mineral surface via long stretches of acidic amino acid residues (Hunter and Goldberg, 1993). In similarity to BSP, OSAD also has long sequences of acidic amino acids and these are situated within the extended C-terminal domain of the molecule. Hence, from the above information, it may be postulated that

OSAD functions in an analogous manner to BSP and facilitates the process of nucleation. The current investigation also localized OSAD within the mineralized dentin, which supports the proposal of a regulatory role for OSAD at the mineral deposition stage. However, in order to fully verify this hypothesis, further functional investigations are warranted. The interaction between OSAD and HAP requires to be fully established and the effect on the regulation of nucleation and subsequent HAP crystal growth determined.

In addition, to the important information that will be obtained following functional investigations on OSAD, the generation of transgenic mice, which lack or over-express the molecule, will also provide vital information as regards elucidation of the roles of OSAD in the mineralization process. However, to date, no reports have been published which have described the effects of OSAD knockout or over-expression in animals. Detailed in this thesis, the knock down effects of gene expression was explored using antisense oligonucleotides, added to cell and organ cultures, in an attempt to evaluate the potential functions of a protein. Although, the antisense oligonucleotides used within this experiment failed to reveal any changes, the value of this technique should not be underestimated in the evaluation of the effects of NCPs on osteogenesis and odontogenesis.

As well the characterization of OSAD in mineralized tissues, this thesis has identified the presence of two Ca^{2+}-binding proteins in bone and dental tissues, Nuc and CRT. Nuc was found to be present both intracellularly, in bone cells and odontoblasts, and extracellularly in the bone and dentin ECM. For the first time, CRT was shown to be a secreted protein, following identification in the predentin matrix. The study also confirmed the intracellular localization of CRT within odontoblasts. It is interesting to note, that Nuc and CRT, which share 60% sequence homology, were found to co-localize in odontoblasts and dentin, thereby suggesting that these two proteins possess similar functions and could potentially act in concert during the biomineralization process.

Mineral deposition in bone and dentin is facilitated by the transport of Ca^{2+} and P_i ions, from the circulation, across the cell layer to the mineralization front. Studies have established that the transport of Ca^{2+} occurs mainly via transcellular mechanisms (Linde and Magnusson, 1975). The detection of Nuc and CRT both intracellularly and extracellularly, suggests that these molecules play key roles in maintaining Ca^{2+} levels, sequestering Ca^{2+} ions to the mineralization front and possessing regulatory roles on the mineralization process. In support of this, previous studies have shown CRT to inhibit the mineralization process in an osteoblastic cell culture system via nuclear hormone receptor-mediated pathways (St-Arnaud *et al.*, 1995) and potentially CRT may be postulated to act in a similar manner in dentin producing cells. Moreover, within the present study, Nuc was also suggested to have a regulatory role in the mineralization process. Low levels of Nuc gene expression were detected during the proliferation of osteoblasts, which was seen to increase prior to the commencement of nodule formation and matrix maturation, but subsequently declined with the progress of mineralization. In order to establish the potential roles for Nuc during mineralization, such as the ability to act as a regulator of HAP nucleation,

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all my friends and colleagues who, in different ways, have helped me and contributed to the completion of this thesis, and especially to:

Mikael Wendel, Managing Director of COB, and my supervisor, for introducing me to the field of matrix proteins, and for being patient, supportive and always taking the time for even the smallest questions, and also for making COB to such an interesting working place.

Björn Klinge, my co-supervisor, for introducing me to the field of bone biology and for always being supportive and genuinely interested in my work.

Dick Heinegård, Head of the Department of Cell and Molecular Biology, Section for Connective Tissue Biology, for accepting me at your lab and for giving me the best possible start in science.

Eszter Somogyi, for being the best friend and roommate ever, for all the great laughs, inspiring talks (about everything but science), great collaboration and much more. There will never be a dull moment in your company!

Rachael Sugars, for bringing enthusiasm to the lab, for being generous, helpful, skilful, friendly and much, much more, but most of all a dear friend. During the preparation of this thesis you have been invaluable!

Marie-Louise Olsson, for introducing me to the field of protein chemistry, for always being very helpful and a true friend, and for nice afternoon coffee-talks.

Cia Christersson, for patiently introducing me to the field of molecular biology and for always being cheerful and helpful.

Carin Lundmark, for sharing your unique expertise in histology and morphological methods and for being a never-ending source of ideas.

Marja Kärki, for all the perfect histological sections and for always being so helpful and positive.

Kjell Hultenby, for nice collaboration and for all the time you spend with our samples.

Carina Krüger, for all nice talks about life and science (in that order), and for sharing with me the worries and laughter of motherhood.

Anna Milan, for nice collaboration, genuine interest in my work and for being a dear friend to the family.

Past and present friends and colleagues at COB, *Thomas Lind, David Lee, Elerin Kärner, Anders Rehn, Kaj Fried, Karin Holmvall, Harold Marcotte, Anna Hultberg, Neha Pant, Pia Hägg Haraldson, Inger Carlsson, Jörgen Jönsson, Anders Krüger, Jonas Eriksson and Emma Stenholm*. You have made every day at the lab a true pleasure!

My parents, *Kjerstin and Roland,* for unconditional love and support and for always taking good care of Adam whenever we needed help, despite the physical distance.

My sister *Catarina* and her family, for inspiring me to start working with research, for love and support and all the relaxing days in Uppsala.

Åke and *Ing-Marie Berg*, my parents-in-law, for support and invaluable help with taking care of Adam

Johan, for all your love and endless patience and support throughout the work of this thesis, and our son *Adam*, for reminding us of what is truly important in life.

This project was supported by grants of the Stockholm County Council, Swedish Research Council and the research funds of Karolinska Institutet.

further studies are required to examine the interaction with other mineralized tissue components.

Taken together, the studies included within this thesis have contributed to the ever-emerging picture of the complex events that occur during the biomineralization of bone and dentin. Further investigations are warranted to elucidate the diverse and significant array of functions that these NCPs identified possess.

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