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The Calcitonin Gene Family of Peptides: Receptor Expression and Effects on Bone Cells

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ABSTRACT

The Calcitonin Gene Family of Peptides: Receptor Expression and Effects on Bone Cells
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The calcitonin gene family of peptides consists of calcitonin (CT), two calcitonin gene related peptides (α-CGRP, β-CGRP), adrenomedullin (ADM), amylin (AMY), three calcitonin receptor activating peptides (CRSP1-3) and intermedin/adrenomedullin2 (IMD). These peptides bind to one of two G protein -coupled receptors, the calcitonin receptor (CTR) or the calcitonin receptor-like receptor (CRLR). The receptor specificity to different ligands is dependent on the formation of a complex with one of three receptor activity-modifying proteins (RAMP1-3).

The aim of this study was to analyse effects of this family of peptides on the formation of osteoclasts and bone resorption, and the expression of the receptor components in bone cells.

CT inhibited the formation of multinucleated osteoclasts in spleen cell cultures and in bone marrow macrophage cultures (BMM) without affecting a number of genes important for osteoclast differentiation, activity or fusion of osteoclast progenitor cells. All members of the CT family, except ADM, inhibited osteoclastogenesis in BMM. The inhibitory effect seemed to involve activation of both protein kinase A and the exchange protein directly activated by cyclic AMP (Epac) signalling. BMM expressed the CRLR, RAMP1-3 and the receptor component protein (RCP). AMY, ADM, CGRP and IMD, but not CRSP and CT, increased cyclic AMP (cAMP) levels in these cells, indicating the presence of functional receptors. Stimulation of BMM with RANKL gradually increased the levels of CTR mRNA as well as the capacity of the cells to respond to the stimulation by CRSP and CT. The response to stimulation of ADM was, on the contrary, decreased by RANKL. Stimulation of RANKL caused a transiently enhanced CRLR mRNA expression and transiently decreased RAMP1, but did not affect RAMP2, RAMP3, or RCP mRNA. However, RANKL did not affect protein levels of CRLR or RAMP1-3. CT, CGRP, AMY, ADM, IMD and CRSP all down regulated the CTR mRNA, but none of the peptides caused any effects on the expression of CRLR or any of the RAMPs.

All members of the CT family, except ADM, rapidly and transiently, inhibited bone resorption in mouse calvarial bones. CT, CGRP, AMY and CRSP also significantly stimulated cAMP formation in the calvaria. cAMP analogues specifically stimulating the PKA or the Epac pathways did not cause inhibition of bone resorption in the calvaria. An unspecific cAMP analogue, stimulating both pathways did, however, cause inhibition.

Analyses of an osteoblastic cell line, MC3T3-E1, showed that these cells express the mRNA for CRLR and all three RAMP proteins.

In conclusion, the results of this thesis show that all peptides in CT family of peptides, except ADM, inhibit of bone resorption and osteoclast formation and that these effects involve the adenylate cyclase-cAMP pathway. Furthermore, expressions of CRLR and RAMP1-3 mRNA have been demonstrated on osteoclasts, as well as in an osteoblastic cell line.

Key words: CT family of peptides, osteoclast differentiation, bone resorption, CTR, CRLR, RAMPs

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PREFACE

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Granholm S, Lundberg P, Lerner UH
Calcitonin inhibits osteoclast formation in mouse hematopoetic cells independently of transcriptional regulation by RANK and c-Fms
J Endocrinol (2007) 195:415-427

II. Granholm S, Lundberg P, Lerner UH
Expression of the calcitonin receptor, calcitonin receptor-like receptor and receptor activity modifying proteins during osteoclast differentiation
J Cell Biochem (2007) In press

III. Granholm S, Lerner UH
Calcitonin receptor-stimulating peptide and intermedin inhibit bone resorption, osteoclast activity and osteoclastogenesis. Manuscript

IV. Granholm S, Lundgren I, Boström I, Lerner UH
Expression of the calcitonin receptor, calcitonin receptor-like receptor and receptor activity modifying proteins in primary osteoblast-like cells.
Manuscript

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ABBREVATIONS

α-MEM	α modification of minimal essential medium
ADM	adrenomedullin
AMY	amylin
BMM	bone marrow macrophages
BMP	bone morphogenic protein
BSA	bovine serum albumin
cAMP	cyclic 3′, 5′ adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene related peptide
CRLR	calcitonin receptor-like receptor
CRL	calcitonin receptor-like receptor
CRSP	calcitonin receptor-activating protein
CT	calcitonin
CTR	calcitonin receptor
D3	α1.25 dihydroxy vitamin D3
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein directly activated by cAMP
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSD	functional secretory domain
GPCR	G protein coupled receptor
IL	interleukin
IMD	intermedin
ITAM	immunoreceptor tyrosine-based activation motif
M-CSF	macrophage-colony stimulating factor
MuOCL	multinucleated osteoclast
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB
OPG	osteoprotegerin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PTH	parathyroid hormone
RAMP	receptor activity modifying proteins
RANK	receptor activator of NFκB
RANKL	RANK ligand
TRAP	tartrate resistant acid phosphatase

INTRODUCTION

Bone

The skeleton is an organ that is built up by bone and cartilage. It functions as a rigid structure which holds up the body as well as protects inner organs. In addition, it serves as attachment points for the skeletal muscles, thus enables movements, and holds the bone marrow in its cavity. Finally, it functions as a mineral supply which is important to uphold the mineral homeostasis in the body fluids.

Bone composition

Bone is a highly specialized form of connective tissue, providing rigidity to the skeleton but with some elasticity remaining. Approximately 25% of bone is made up by an organic matrix, of which 90-95% consists of collagen type I. The remaining 5-10% of the matrix is composed by proteoglycans and other non-collagen proteins. The collagen is arranged in fibrils, which are further arranged into networks (Rossert & de Crombrugghe, 2002). Embedded in the organic matrix are inorganic minerals, mainly calcium and phosphate in the form of hydroxyapatite crystals, which constitute about 70% of the bone mass. These hydroxyapatite crystals coat the collagen fibrils, providing rigidity to the tissue (Weiner & Traub, 1992). The remaining 5% of the bone tissue comprises of water and bone cells.

There are two morphologically different types of bone: cortical (compact) and trabecular (cancellous, spongy). Cortical bone, which represents 80% of the bone mass, is arranged in concentric lamellae. These lamellae are arranged in perpendicular planes, providing density and strength to the bone. The trabecular bone has a more spacious structure forming a network, throughout the bone marrow. The structural differences between the two bone types are related to their primary functions: cortical bone should withhold mechanical stress, whereas trabecular bone, with a higher surface per bone unit has a more pronounced metabolic function (Marks & Odgren, 2002). The inside of the cortical bone, as well as trabecular bone, are covered by the endosteum which separates the bone surfaces from the bone marrow. The outer surface of the cortical bone is covered by the periosteum, separating the bone from the surrounding tissues. The periosteum conisist of two layers of cells. The outer layer contains fibroblastic cells, collagen and networks of nerves and blood vessels, whereas the inner layer have a higher density of cells, including bone cells, fibroblasts and nerve cells (Allan et al., 2004).

Embryonic bone formation

The embryonic development of the skeleton is conducted through two different processes: the endochondral ossification and the intramembranous ossification. Both processes begin with the condensation of embryonic mesenchymal cells, defining the position for the formation of the bone. In *the endochondral ossification*, the mesenchymal cells differentiate into chondrocytes that forms a model of the bone in cartilage, which is subsequently mineralized. The mineralized cartilage is thereafter invaded by blood vessels together with osteoblasts and chondroclasts from the surrounding tissues. The mineralized cartilage matrix is then degraded and replaced by bone matrix, produced by the osteoblasts. An area of cartilage is, however, maintained at the bone ends, to facilitate additional bone growth throughout childhood and puberty. This area is called the epiphyseal plate and will remain until after puberty, after which it is lost (Nakashima et al., 2002; Kronenberg, 2003; Walsh et al., 2006). *The intramembranous ossification* occurs in flat bones and in this process, the cells in condensation differentiate directly into bone-forming osteoblasts. The osteoblasts secrete bone matrix, resulting in the formation of bone islands. These islands increase in size, growing towards each other and eventually meet at the sutures. Sutures are composed by periost of adjacent bones. At the center of the sutures, there is a proliferating cell population, able to differentiate into osteoblasts (Marks & Odgren, 2002).

The Osteoblast

Osteoblasts origin from a mesenchymal pluripotent stem cell that can also differentiate into chondrocytes, fibroblasts, tendon cells and adipocytes. Osteoblasts are responsible for the synthesis of the bone tissue, a process carried out in two steps. First, the osteoblasts produce the organic collage-rich bone matrix called the osteoid and this osteoid is thereafter mineralized. However, closest to the bone surface, a layer of unmineralized osteoid always remains. Active osteoblasts have a cuboidal structure, and since they produce a variety of proteins they have a very pronounced Golgi apparatus and endoplasmatic reticulum. Osteoblasts are very similar to fibroblasts, which also are capable of matrix production. But, whereas fibroblasts release of matrix is pericellular, the osteoblastic secretion is polarized, towards the bone surface. In addition, osteoblasts express two genes, which are essential for osteoclast survival and differentiation, *csf1* and *tnf11*, coding for the macrophage-colony stimulating factor (M-CSF) and the receptor activator of nuclear factor κB ligand (RANKL), respectively. Osteoblasts also express a third gene, (*tnfrsf11b*) coding for osteoprotegerin (OPG), a soluble TNF receptor that functions as a decoy receptor for RANKL. The importance of these three genes is discussed under the osteoclast section.

Transcription factors involved in osteoblast differentiation

Core binding factor 1/runt related transcription factor 2

The first transcription factor involved in osteoblast differentiation to be discovered was the core binding factor 1/runt related transcription factor 2 (cbf1/runx2). In 1997, Ducy et al. (1997) characterized a region in the osteocalcin promoter, which they termed OSE2. The factor binding to this region was a member of the cbf1 family of transcription factors, the mouse homologue to Runt in *Drosophila melanogasker* (runx). Concurrently, it was observed that in mice lacking Runx2, osteoblast differentiation is arrested and the skeleton consists only of cartilage (Komori et al., 1997; Otto et al., 1997). Runx2 have been shown to be both essential and sufficient for both osteoblast and chondrocyte differentiation. It is constantly expressed, in differentiating as well as in mature osteoblast. Not much is known about the regulation of runx2, but several studies show that Wnt/Lrp5 signalling is involved (Komori, 2006). During skeletal development, the expression of *runx2* is upregulated several days before osteoblast differentiation occur. This delay is dependent on two nuclear proteins, Twist-1 (in craniofacial skeleton) and Twist-2 (appendicular skeleton), that bind to DNA and thereby inhibit the activation of transcription by runx2. Schnurri 3, a zink finger protein, is also involved in regulation of runx2, by recruiting WWP1, the E3 ubiquitin ligase, thereby endorsing runx2 degradation. Runx2 also stimulates the differentiation of hyperthrophic chondrocytes, which precede the replacement of cartilage by bone. (Reviewed by Karsenty, 2007)

Osterix

A gene immediate downstream of runx2 is *osterix*. Runx2 deficient mice do not express osterix, whereas mice lacking osterix do express runx2. Osterix deficient mice have no osteoblasts and have downregulated expressions of several osteoblastic genes, such as collagen α1, bone sialoprotein, osteopontin and osteocalcin (Nakashima et al., 2002). In mice deficient of osterix, both intramembranous and endochondral ossification is hampered but the cartilage growth plate is normal (Nakashima et al., 2002). Mice lacking osterix have normal chondrocytes and it therefore seems as runx2 is the common transcription factor for both osteoblasts and chondrocytes, whereas osterix is specific for osteoblast differentiation.

The canonical Wnt/Lrp5 signalling

The Wnt proteins are the homologues to wingless in *Drosophila melanogasker*. This family consists of 19 members that bind to a membrane receptor complex of a G protein-coupled receptor (Frizzled) and a low-density lipoprotein receptor-related protein (LRP). Binding of a Wnt protein to the Frizzled/LRP receptor complex leads to the transduction of signals to several intracellular proteins. The best characterized pathway is the Wnt/β-catenin signalling through LRP5, often referred to as the canonical Wnt signalling pathway. β-catenin is a transcriptional regulator, and in the absence of Wnt signalling this protein is rapidly degraded

and the cytoplasmic levels of β-catenin are low. When the cells receive Wnt signals, degradation is inhibited, and consequently the intracellular levels of β-catenin are increased. Nuclear β-catenin interacts with transcription factors of the Lef/Tcf family to regulate transcription of Wnt target genes. The canonical Wnt signalling is reviewed by Logan & Nusse (2004).

The importance of the canonical Wnt signalling in osteogenesis has been shown by several genetically modified mice. Mice deficient of *Lrp5* suffer from pseudoglioma syndrome, characterized by early onset osteoporosis and blindness (Kato et al., 2002; Fujino et al., 2003; Holmen et al., 2004). In addition, embryos from β-catenin conditional knock-out mice lacked bone, although cartilage was formed (Hu et al., 2005), as a consequence of arrested osteoblast differentiation. Recently, β-catenin has been shown to be important in regulation of *tnfrsf11b* (coding for OPG) expression, thereby controlling osteoclast differentiation and subsequently bone resorption (Glass II & Karsenty, 2006).

Nuclear factor of activated T cells

Nuclear factor of activated T cells 2 (NFAT2, also known as NFATc1) belongs to a family of transcriptor factors initially identified in T cells during activation of the immune system (Shaw et al., 1988), and have later been identified as the most important transcription factor during osteoclastogenesis (Ishida et al., 2002; Takayanagi et al., 2002). Inactive NFAT2 is highly phosphorylated and is retained in the cytoplasm. Upon dephosphorylation by calcineurin, NFAT2 is activated and translocates into the nucleus. Transgenic mice, in which osteoblasts have constitutively high levels of nuclear NFAT2, have an increased number of both osteoblasts and osteoclasts (Winslow et al., 2006). NFAT2 have been shown to form a DNA binding complex with osterix, thereby cooperating with this transcription factor in control of osteoblast differentiation (Koga et al., 2005). Signalling of NFAT in osteoblasts leads to enhanced chemochine expression which may attract more osteoclast precursor cells to the bone surface and thereby enhance osteoclast formation (Winslow et al., 2006).

The Osteocytes and the bone lining cells

Approximately 90% of the cells in the bone tissue are osteocytes. These cells are terminally differentiatied osteoblasts which have been embedded in the bone matrix. Osteocytes are poor in organelles, suggesting that their main function is no longer matrix synthesis. These cells are connected to each other and to osteoblasts on the bone surface via long extensions, canaliculi, which enables communication between cells (Knothe Tate et al., 2004). The function of osteocytes is not fully understood but they are believed to act as mechanosensors in bone tissue, which allow bone cells to respond to environmental changes. Also, apoptotic osteocytes increase the secretion of osteoclastogenic cytokines,

thereby enhancing bone resorption (Noble et al., 2003). The bone lining cells are flat, elongated, inactive osteoblastic cells that cover the bone surfaces. Not much is known about these cells but it has been suggested that they are responsible for initiation of bone remodelling, by matrix degradation (reviewed by Rauner et al., 2007).

The Osteoclast

The osteoclast is of hematopoetic origin, derived from the monocyte lineage (Takahashi et al., 2002). The fate of this progenitor cell is dependent on extracellular stimuli, i.e., stimulation by GM-CSF favours the differentiation into dendritic cells, M-CSF stimulates macrophage proliferation and differentiation, whereas M-CSF and RANKL promotes osteoclast development. There are three hematopoetic organs: the bone marrow, the spleen and during embryogenesis the fetal liver. The progenitor cells are found in the circulation before attracted to the tissue were terminal differentiation into osteoclasts or macrophages occurs.

The osteoclast is a multinucleated cell, formed by the fusion of several mononucleated precursor cells. Its function is to degrade old mineralized bone matrix, by a complex mechanism, discussed under the remodelling cycle section.

Differentiation of osteoclasts

The differentiation of osteoclast progenitor cells into active osteoclasts requires the activation of several intracellular signalling programs. Two of these signalling patways, activation av c-Fms and RANK, are indispensible for osteoclastogenesis to occur. More recently, the importance of signalling by immunoreceptors complexes have also been shown. Some of the, currently known, most important signalling pathways, activated during osteoclastogenesis are shown in fig. 1.

Macrophage colony stimulating factor

Proliferation and survival of the common myeloid precursor cells is dependent on the macrophage colony stimulating factor (M-CSF, also called CSF-1). M-CSF was first isolated from fetal yolk sac (Johnson & Metcalf, 1978), and shown to stimulate bone marrow granulocyte-macrophage progenitor cells (GM-CFC) to preferentially differentiate into macrophages (Johnson & Burgess, 1978). The importance of M-CSF in osteoclast formation was later confirmed by the discovery that mice deficient in full-length M-CSF (*op/op*), due to a mutation in the *cfs1* gene (Yoshida et al., 1990) suffered from severe osteopetrosis and lacked osteoclasts (Wiktor-Jedrzejczak et al., 1990). The osteopetrosis of *op/op* mice could be rescued by administration of soluble full-length M-CSF. M-CSF is expressed by several cell types, including endothelial cells, fibroblasts,

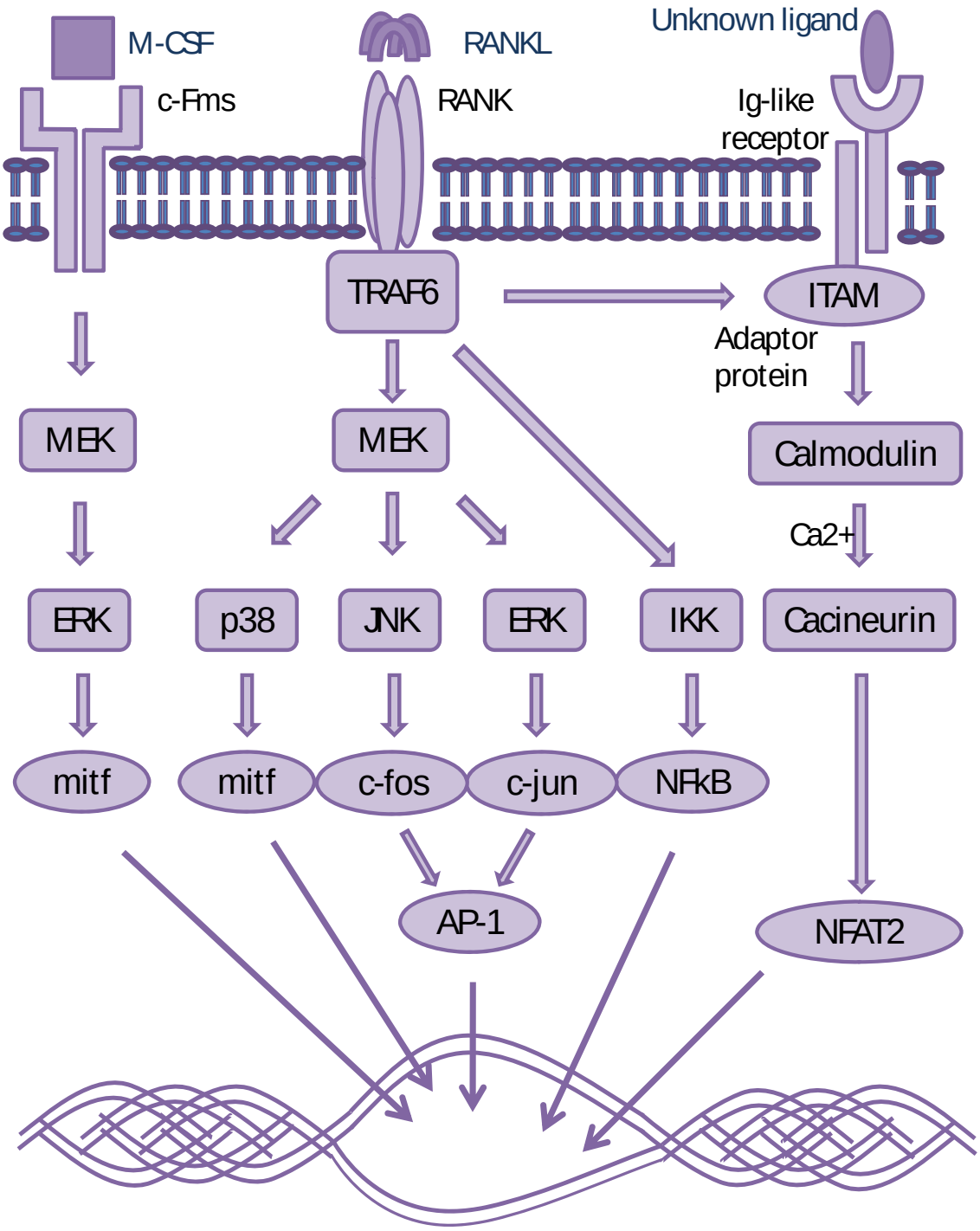

Figure 1. Intracellular signalling pathways involved in osteoclast differentiation

monocytes as well as osteoblasts and stromal cells. Due to differential splicing and post-translational modifications, M-CSF exists in several isoforms; both membrane-bound and secreted (Stanley et al., 1997) although the soluble form has been reported to be required for osteoclastogenesis (Dai et al., 2004).

The receptor for M-CSF is c-Fms and is expressed on early monocyte progenitor cells. c-Fms is the gene product for the proto-oncogene *csf1r* (Sherr et al., 1985). The expression of c-Fms is positively regulated by the transcription factor PU.1, which binds to the *csf1r* promoter region. Mice lacking PU.1 (Tondravi et al., 1997) as well as mice lacking *csf1r* (Dai et al., 2002), both exhibit similar phenotypes as the *op/op* mouse, with osteopetrosis due to deprivation of osteoclasts progenitor cells. c-Fms has an intrinsic tyrosine kinase activity and upon ligand binding, c-Fms homodimerizes and autophosphorylation occur on selected tyrosine residues. These phosphorylated residues function as binding sites for proteins containing a SH2 domain, e.g., Grb2 and c-Src, which then transduce the signal and activates pathways such as extracellular signal-regulated protein kinases (ERK1/2) and PI3K (reviewed by Ross, 2006). One of the genes downstream of c-Fms is the transcription factor MITF. MITF binds to the promoter, and thereby induce the expression, of the anti-apoptotic protein Bcl-2. Both MITF- and Blc-2 deficient mice have an osteopetrotic phenotype (McGill et al., 2002). Another important effect of M-CSF stimulation of the myeloid precursor cells is the upregulation of receptor activator of NFκB (RANK).

Receptor activator of NFkB ligand

Whereas activation of c-Fms has been shown to be crucial for proliferation and survival of the common myeloid progenitor cells, the activation of RANK has been shown to be essential for further differentiation of the progenitors into osteoclasts (reviewed by Teitelbaum, 2000; Teitelbaum & Ross 2003; Lerner, 2004; Asagiri & Takayanagi 2007). RANKL binds to RANK and deficiency in any of these results in loss of multinucleated osteoclasts, without affecting the number of progenitor cells (Theill et al., 2002). OPG is the third component of this system, functioning as a soluble decoy receptor binding to RANKL, thereby inhibiting RANK/RANKL interaction.

OPG was discovered simultaneously by several different groups (Simonet et al., 1997; Tan et el., 1997; Tsuda et al., 1997; Yun et al., 1998) as an inhibitor of osteoclast formation. The importance of OPG as a regulator of bone density has been illustrated by mice lacking OPG, as well as in transgenic mice overexpressing OPG. The deletion of OPG results in severe early-onset osteoporosis, with decreased density in both cortical and trabecular bone (Bucay et al., 1998; Mizuno et al., 1998; Yasoda et al., 1998a). Overexpression of OPG, on the contrary, led to an osteopetrotic phenotype, with increased bone mineral density and a reduced number of osteoclasts (Simonet et al., 1997).

OPG is a member of the TNF receptor superfamily. Unlike the other members of this family, OPG lacks both a transmembrane region and a cytoplasmic domain and therefore only exists in a soluble form. The amino terminal of OPG contains four cysteine-rich domains which are important for ligand binding. OPG is expressed in a variety of tissues, including osteoblasts, stromal cells, fibroblasts, and endothelial cells.

RANKL was also discovered in the late 1990s, as a protein that could bind to OPG (Lacey et al., 1998) and induce osteoclast formation (Yasoda et al., 1998b). The peptide was identical to a protein that could stimulate T cell growth (Anderson et al., 1997) and to TNF-related activation induced cytokine (TRANCE), discovered by Wong et al. (1997) as a protein that bound to TNF receptors on T cells. RANKL is a member of the TNF superfamily of cytokines and is expressed by, among others, osteoblasts. It exists both as a membrane-bound and a soluble form and the different forms are a result of proteolytic shedding of the RANKL ectodomain (Lum et al., 1999). RANKL forms a homotrimer with four receptor binding loops in the extracellular domain, to which homotrimerized RANK bind (Lam et al., 2001; Ito et al., 2002).

The importance of RANKL in osteoclast differentiation has been demonstrated by both RANKL knock-out mice (Kong et al., 1999) and mice overexpressing the soluble form of RANKL (Mizuno et al., 2002). RANKL knock-out mice exhibited an osteopetrotic phenotype whereas mice overexpressing RANKL showed an osteoporotic phenotype. Both these animals had affected number of osteoclasts. RANKL deficiency resulted in a decreased number of osteoclasts whereas the number was increased in overexpressing mice. RANKL deficiency also affected T and B cell differentiation, and the development of lymph nodes (Kong et al., 1999).

RANK is expressed on osteoclast progenitor cells and is the receptor for RANKL (Hsu et al., 1999; Li et al., 2000). RANK, like OPG, is a member of the TNF receptor superfamily and has four cystein-rich domains in the extracellular amino terminal, necessary for ligand recognition and binding (Locksley et al., 2001). The signalling cascade following activation of the receptor is mediated by TNF receptor associated proteins (TRAFs), which bind to the intracellular portion of the receptor (reviewed by Arch et al., 1998). RANK signalling is predominately mediated by TRAF6 (Galibert et al., 1998; Darney et al., 1999) and activates several intracellular signalling pathways, mediated by mitogen-activated protein (MAP) kinases: c-Jun –N-terminal kinase (JNK), ERK1/2, p38 and c-Src. Eventually, activation of RANK leads to the activation and translocation into the nucleus of several transcription factors, including nuclear factor κB (NFκB), activator protein-1 (AP-1) and NFAT2, the most important transcription factor involved in osteoclast differentiation. RANK deficient mice suffer from severe osteopetrosis due to a complete lack of osteoclasts (Dougall et al., 1999; Li et al.,

2000). Similarly, mice lacking TRAF6 also exhibit the osteopetrotic phenotype (Lomaga et al., 1999; Naito et al., 1999).

Immunoreceptors and co-stimulation

RANKL has been shown to be indispensible for osteoclast formation and RANKL stimulation leads to a massive increase of NFAT2 expression. However, induction of NFAT2 involves dephosphorylation by calcineurin, which is activated by Ca^{2+} and RANKL, being a member of the TNF receptor family, is not related to Ca^{2+} signalling. This indicated the presence of some additional signal, linking these two events.

DAP12 and Fc receptor common γ chain (FcRγ) are adaptor proteins that form complexes with a number of different immunoglobulin-like (Ig-like) receptors in B cells, T cells and natural killer cells. These adaptor proteins have an immunoreceptor tyrosin-based activation motif (ITAM) in the intracellular part and upon receptor activation of the receptor, the ITAM motif is phosphorylated and recruits Syk tyrosine kinase. This results in the activation of phospholipase Cγ and subsequent Ca^{2+} signalling (Mócsai et al., 2004; Takayanagi, 2005). Mice deficient in DAP12 exhibit mild osteopetrosis but a normal number of osteoclasts, whereas the FcRγ knock-out do not have any phenotypic differences from the wild-type. Mice deficient in both DAP12 and FcRγ exhibit a severe osteopetrotic phenotype *in vivo* and inhibition of osteoclast formation *in vitro* (Kaifu et al., 2003; Koga et al., 2004). These results indicate that ITAM signalling is required for osteoclastogenesis to occur and suggest that activation of this system could be "the missing link" that connects RANKL stimulation to the activation of NFAT2. Ig-like receptors identified in osteoclast progenitor cells include osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor A (PIR-A), signal-regulatory protein 1β (SIRP1β) and Triggering receptor expressed on myeloid cells 2 (TREM2; Kim et al., 2002; So et al., 2003; Koga et al., 2004). It has later been shown that RANK signalling induces phosphorylation of the ITAM motif of both DAP12 and FcRγ. Since ITAM signalling have been shown to be essential for the induction of NFAT2 during osteoclastogenesis, but cannot induce osteoclastogenesis alone, they should be called co-stimulatory to RANK.

Transcription factors involved in osteoclast differentiation

Nuclear factor of activated T cells 2

The role of NFAT2 in osteoclastogenesis was discovered by large scale gene expression analyses and it was shown to be a key regulator of osteoclast differentiation (Ishida et al., 2002; Takayanagi et al., 2002). Upon stimulation by RANKL, transcription of NFAT2 is extensively upregulated. The initial induction of NFAT2 is activated by pre-existing NFAT1/NFATc2, in cooperation with other transcription factors such as NFκB and AP-1, which binds to the promoter region of NFAT2 and activates transcription. In addition, NFAT2

binds to its own promoter, thereby causing an autoamplification of its own gene expression (Asagiri et al., 2005). Deficiency of NFAT2 cause embryonic lethality, but Asagiri et al. (2005) were able to show that chimeric mice, where $NFAT2^{+/+}$ embryonic stem cells were injected into blastocysts derived from osteoclast deficient $Fos^{-/-}$ mice, were rescued from osteopenia and developed normal osteoclasts. In contrast, $NFAT2^{-/-}$ embryonic stem cells could not restore the osteopetrotic phenotype of Fos deficient mice. These results clearly show that NFAT2 is essential for osteoclast formation. The genes regulated by NFAT2, in cooperation with other transcription factors, include cathepsin K, tartrate resistant acid phosphatase (TRAP), integrin β_3 and the calcitonin receptor.

Nuclear factor of κB

The NFκB family consists of several dimeric transcription factors. There are five members of this family: RelA, RelB, c-Rel, p50 and p52. Inactive NFκB is present in the cytosol, bound to an inhibitor (inhibitor of NFκB, IκB). IκB binds to a region called the Rel homology region, important for dimerization and which also contains a nuclear localization sequence. Upon extracellular stimuli, an IκB kinase, (IKK) is activated and phosphorylates IκB on two conserved cystein residues. The phosphorylated IκB is recognized by ubiquitine conjugating enzymes and upon ubiquitination, it is degraded by the 2ES proteasome complex. The dissociation of NFκB from its inhibitor reveals the nuclear localization sequence and leads to translocation of the transcription factor into the nucleus where it binds to the κB site in the promoter of its target genes. The importance of NFκB in osteoclast development have been shown by generation of knock-out mice. The p50/p52 double knock-out mouse suffer from severe osteopetrosis and a complete lack of both mono- and multinucleated TRAP positive cells (Franzoso et al., 1997). Mice deficient in only one of these subunits did, however, not exhibit any phenotype, suggesting that p50 and p52 have redundant functions.

Activator protein-1

AP-1 are dimeric transcription factors, composed mainly of members of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) families of proteins. Whereas Jun proteins only can form heterodimers with Fos proteins, the Fos proteins can form both hetero- and homodimeric complexes, capable of inducing transcription of their target genes.

Mice lacking c-Fos (Johnson et al., 1992; Wang et al., 1992) are viable and fertile but suffer from osteopetrosis due to a lack of osteoclasts. In $Fos^{+/+}$ mice, but not in $Fos^{-/-}$ mice, NFAT2 is induced during osteoclastogenesis, indicating that c-Fos is important for NFAT2 induction (Matsuo et al., 2004). In addition, Matsuo et al. (2004) showed that the in osteoclasts derived from $Fos^{-/-}$ mice, exogenous NFAT2 expression could restore the expressions of TRAP and CTR.

c-Jun is a protein forming an AP-1 complex with c-Fos. Mice deficient in c-Jun are embryonic lethal, but Ikeda et al. (2004), using transgenic mice expressing a dominant negative form of c-Jun in osteoclasts, have shown that the bone marrow cavities of these animals are filled with unresorbed bone. In addition, these animals have a decreased number of osteoclasts in long bones. These results show that not only c-Fos, but also c-Jun is essential for normal osteoclastogenesis to occur.

MafB

MafB is a family of basic region, leucin zipper (bZIP) motif containing, DNA binding proteins. The family of MafB is divided into two groups, MafB-large, containing a transactivating domain as well as the bZIP, and MafB-small, containing only the bZIP region, needed to form dimeric complexes. MafB is expressed in monocytes and have been shown to stimulate macrophage formation (Sieweke et al., 1996). Recently, Kim et al. (2007) have reported that MafB is downregulated during RANKL induced osteoclast differentiation and that retroviral overexpression of MafB in BMM cultures, inhibits the RANKL induced formation of multinucleated osteoclasts, by binding to the promoter region of target genes for the transcription factors c-Fos and NFAT2.

The bone remodelling cycle

Beyond embryonic development, the skeleton is constantly remodelled in response to regulatory signals, to adapt to changing requirements of the body, such as release of mineral into the bloodstream, increased mechanical stress or repairs of micro damages to the skeleton. The remodelling process occurs in two separate events: the bone resorption by the osteoclast and the subsequent bone formation of the osteoblast. When these two events occur in concordance the bone mass stays constant, but if this equilibrium is disturbed the result can be either an increased bone resorption and/or a decreased bone formation or vice versa. Whereas the resorption process takes 3-4 weeks, the bone formation takes several months, and therefore these two events needs to be tightly regulated, a process called coupling. Impairment of coupling can lead to pathological conditions with decreased bone mass such as osteoporosis, or conditions with increased bone mass such as osteopetrosis. The remodelling cycle is shown in fig. 2.

The bone remodelling process is initiated by the activation of inactive osteoblasts and bone lining cells, by several signals such as systemic hormones, growth factors and cytokines as well as by decreased loading. The activated osteoblasts then starts producing and secreting proteolytic enzymes, degrading the osteoid (Vaes, 1988), and exposing the mineralized bone matrix.

Another important function of the activated osteoblasts is to attract osteoclast precursor cells from the blood stream to the resorptive site, by a "homing process", however the mechanism underlying this process is not fully understood. When the osteoclast precursor cells enter the resorptive area, they are stimulated by the osteoblasts to start differentiating into mature, multinucleated osteoclasts. This process requires cell-to-cell contact (Udegawa et al., 1989) and involves the stimulation by M-CSF and binding of RANKL, expressed by the osteoblasts, to RANK, expressed on the osteoclast precursor cells.

The multinucleated osteoclasts attach very tightly to the mineralized bone matrix, thereby creating an area, the Howship's lacunae, isolated from the surrounding tissue. The attachment is accomplished via integrins α_v and β_3 (the vitronectin receptor), that binds to proteins such as osteopontin, vitronectin and bone sialoprotein in the matrix (Nesbitt et al., 1993). Rearrangements of the cytoskeleton lead to the formation of a dense actin ring at the periphery of the osteoclast membrane, creating the sealing zone, separating the Howship's lacuna from the surrounding tissue. After attachment, the osteoclast undergoes intracellular changes, becoming polarized with different distinct domains such as the ruffled border and an additional functional secretory domain (FSD) at the basolateral domain. The resorption of bone matrix occurs in Howship's lacuna. Cytoplasmic acidic vacuoles fuse with the membrane within the resorption lacunae, thereby creating the ruffled border, and the release of acid into the resorption area provides an acidic environment. In addition, ATPases, located in the ruffled border, transport protons into the resorption lacuna. The vacuolar-type H^+-ATPase (v-ATPase) plays an important role in acidification of the resorption lacuna, and defects in *tcirg1*, the gene encoding for the α3 subunit of v-ATPase, ATP6i, have been shown cause infantile malignant osteopetrosis (Kornak et al., 2000). In addition, it has been shown that the d2-subunit of the v0 domain of v-ATPase is important in bone metabolism. Mice deficient in this subunit have a decreased number of multinucleated osteoclasts (Lee et al., 2006). The protons are supplied by the enzyme carbonic hydrase II, catalyzing the reaction of water (H_2O) and carbon dioxide (CO_2) resulting in the formation of protons (H^+) and bicarbonate (HCO_3^-). The HCO_3^- is transported into the extracellular space via HCO_3^-/Cl^- exchangers in the basolateral FSD. The imported chloride ions are pumped into the resorption lacuna, by specific chloride channels, such as ClC-7 (Kornak et al., 2001). The chloride ions and protons transported into the resorption lacuna forms hydrochlorid acid, and provide an even more acidic environment with a pH of about 4.5. At this pH, the mineral crystals embedded in the organic bone matrix rapidly dissolve.

The next step in the resorption process involves release of various enzymes into the resorption lacuna. These enzymes include the cystein proteinase cathepsin K, matrix metalloproteinase 9 (MMP-9) and TRAP. Whereas cathepsin K and MMP9 degrade the organic matrix, the function of TRAP in this process is not

clear. It has, however, been shown that mice deficient in TRAP exhibit a mild osteopetrotic phenotype, observed already at 4 weeks of age (Hayman et al., 1996; Hollberg et al., 2002) whereas mice overexpressing TRAP have an increased bone turnover (Angel et al., 2000).

The FSD, at the basolateral domain of the membrane, is connected to the ruffled border via microtubules. It has been suggested that these connections are used to transport exocytotic vesicles, containing degradation products which is to be secreted into the extracellular space at the FSD. For review of the resorption process see Väänänen (2005).

During the preceding bone formation, several growth factors such as insulin growth factor I (IGF I) and 2 (IGF II) and transforming growth factor β (TGF-β) was embedded in the bone matrix. During the bone resorption process these factors are released from the matrix and are believed to function as autocrine coupling factors, attracting nearby osteoblasts to the resorption pit. These osteoblasts are then stimulated to form new bone (Rodan, 1991) and the remodelling cycle is thereby completed.

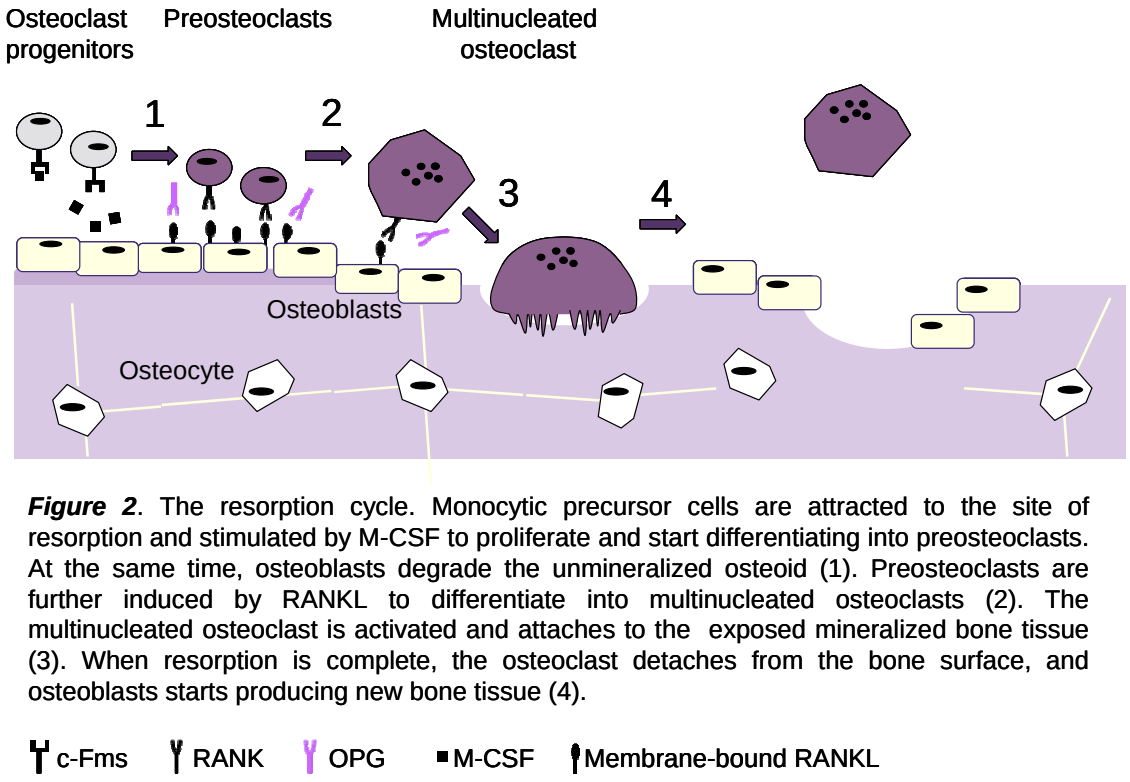

Figure 2. The resorption cycle. Monocytic precursor cells are attracted to the site of resorption and stimulated by M-CSF to proliferate and start differentiating into preosteoclasts. At the same time, osteoblasts degrade the unmineralized osteoid (1). Preosteoclasts are further induced by RANKL to differentiate into multinucleated osteoclasts (2). The multinucleated osteoclast is activated and attaches to the exposed mineralized bone tissue (3). When resorption is complete, the osteoclast detaches from the bone surface, and osteoblasts starts producing new bone tissue (4).

Regulators of bone metabolism

Bone remodelling is not only regulated by cell-cell contact and paracrine stimulation. Humoral factors such as parathyroid hormone, vitamin D, estrogen and calcitonin are well known effectors of bone metabolism. During recent years it has become more and more evident that there is interplay also between bone metabolism and the nervous system, as well as between bone and the immune system. In this section, is given a few examples of the interplay between bone cells and the different regulatory systems of the body.

Hormones

Parathyroid hormone

Parathyroid hormone (PTH) is one of the most important regulators of Ca^{2+} homeostasis. In response to low serum Ca^{2+}, it is secreted from the parathyroid glands into the circulation. PTH has two sites of action: the kidneys and bone. In the kidney it stimulates the re-absorption of Ca^{2+}. In addition it stimulates 1-hydroxylase, the enzyme responsible for converting 25-hydroxy vitamin D3 to its active form, α1.25dihydroxy vitamin D3 (D3). In bone it indirectly stimulates bone resorption by stimulating the expression of RANKL by the osteoblasts. In this sense, PTH has a catabolic effect on bone. However, under some circumstances PTH instead has an anabolic effect, promoting bone formation instead of resorption and PTH is even used to treat osteoporosis. Several studies show that if PTH is administered intermittent, the effect is an increased bone formation, whereas continuous infusion of PTH results in bone loss (Ma et al., 2001; Locklin et al., 2003). The molecular mechanism behind these contradictive actions of PTH are not known (reviewed by Qin et al., 2004; Rosen et al., 2004)

Vitamin D

Vitamin D is a steroid hormone, which is either synthesized in the epidermis or taken up from food. The hormone is converted into its active form, D3, by sequential hydroxylation in the liver and then in the kidney. The receptor for D3, VDR, is expressed in most tissues, indicating that the hormone may be involved in numerous biological actions. VDR is a ligand-activated transcription factor and binding of D3 activates the receptor which then binds to vitamin D responsive elements (VDRE), in the promoter region of its target genes. The most important physiological role of D3, is as a regulatior of Ca^{2+} uptake in the intestine. The effects of D3 in bone metabolism are somewhat diverse. It was first implicated as an inducing factor for bone resorption in “the Raisz assay”, where D3 stimulation resulted in the release of ^{45}Ca from pre-labelled fetal long bones (Raisz et al., 1972). Now, it is established that D3/VDR stimulates osteoclast differentiation indirectly by stimulating RANKL expression of the osteoblasts. In 2000, Endo et al. (2000) reported that under certain conditions, D3

can also inhibit bone resorption. Later, it has been shown that D3/VDR suppress c-Fos protein in osteoclast precursor cells, thereby inhibiting the expression of AP-1 induced genes, important for osteoclast differentiation (Bikle, 2007; Ikeda, 2007).

Estrogen

The systemic hormone estrogen is one of the most important inhibitors of bone resorption. The pronounced effects of estrogen deficiency are clearly illustrated by the observation that ovariectomized mice exhibit an osteoporotic phenotype. Recently, it has been shown that an osteoclast-specific knock-out of the estrogen receptor α (ERα) results in mice with a significant trabecular bone loss, without effect on cortical bone (Nakamura et al., 2007). The same phenotype was seen in ovariectomized wild-type mice, but the osteoporosis in these mice could be reversed by administration of estrogen. In addition, estrogen administration to wild-type mice resulted in an induction of an apoptotic signal, FasL, in osteoclasts. These results suggest that the osteoprotective role of estrogen, in part, is due to induction of apoptosis of osteoclasts (Nakamura et al., 2007). In addition, estrogen deficiency results in increased RANKL and decreased OPG expression in osteoblastic cells, thereby supporting osteoclastogenesis and hence bone resorption (reviewed by Rauner et al., 2006).

The nervous system

Clinical observations of the association between head trauma or stroke, and effects on the morphologic phenotype of bone have long suggested that the nervous system may have an important role in regulation of bone metabolism.

The last decades, it has become evident that there are a number of nerve fibers in the vicinity of bone, as well as in the bone marrow and periosteum. A vast number of neuropeptides, including vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), have been identified and shown to have effect on bone metabolism. VIP has been shown to inhibit D3-induced osteoclastogenesis in bone marrow cultures (Mukohyama et al., 2000) as well as the activity of multinucleated osteoclasts (Lundberg et al., 2000). The importance of VIP as a regulator of bone metabolism is also indicated by the findings that destruction of VIP containing nerve fibers lead to a 50% increase in osteoclast-covered surface in the mandible and calvaria (Hill & Elde, 1991). CGRP is known to stimulate osteoblast proliferation (Cornish et al., 1999) and has also been shown to inhibit D3-induced osteoclastogenesis in bone marrow cultures (Cornish et al., 2001).

In addition to the peripheral control of the nervous system, there is accumulating evidence of a central, hypothalamic regulation of bone metabolism. In 2000,

Ducy et al. (2000) showed that leptin-deficient mice (*ob/ob*) exhibited a high bone mass, due to an increased bone formation rate. That this was a result of central regulation was shown by that infusion of small doses of leptin, which had no effect when administered peripherally, rescued the bone phenotype in *ob/ob* mice when administered centrally. Later, the same group showed that this central regulation by the hypothalamus was conducted by the sensory nervous system and affected osteoblasts via β2-adrenergic receptors (Takeda et al., 2002). More recently, Baldock et al. (2002), have shown that conditional deletion of hypothalamic neuropeptide Y2 receptors cause in increased bone formation rate and higher bone mass. Later, it has also been shown that conditional knock-out of the hypothalamic Y2 receptors also can prevent bone loss, induced by deficiency of sex hormones in gonadectomized mice (Allison et al., 2006).

Osteoimmunology

Cytokines

The interplay between bone metabolism and the immune system was first observed in the 70s when an unknown soluble factor, secreted from activated immune cells where shown to stimulate bone resorption (Horton et al., 1972). This factor was later shown to be interleukin 1 (IL-1) (Dewhirst et al., 1985). Since then, it has become clear that several cytokines, secreted by immune cells, influence the differentiation and activity of bone cells.

Stimulators of bone resorption

IL-1, IL-6, IL-11, OSM, LIF, IL-17 and TNF-α are all considered to be osteolytic cytokines because of their bone resorptive effects *in vivo*. IL-1 is produced by a variety of cells, including macrophages. IL-1 has been shown to stimulate the osteoclastic expression of TRAF6, which is essential for relaying the intracellular signalling following RANK stimulation, and thereby, IL-1 facilitates osteoclastogenesis (Boyle et al., 2003; Suda et al., 2003). IL-1 can also influence osteoclast formation indirectly, by stimulating prostaglandin production and increase the expression of RANKL in the osteoblasts (Suda et al., 2003). IL-6, IL-11, OSM and LIF are closely related and are often referred to as "the IL-6 family of cytokines" (Palmqvist et al., 2002). IL-6 is produced by macrophages, as well as by osteoblasts and stromal cells. In osteoblasts, IL-6 production is induced by PTH and TNF-α, (Dai et al., 2006).

Inhibitors of bone resorption

IL-4, IL-10, IL-12, IL-13, IL-18, IFN-β and IFN-γ are mainly produced by lymphocytes and macrophages, and have all been shown to inhibit bone resorption. Palmqvist et al. (2006) have shown that in bone marrow macrophage cultures, addition of IL-4 or IL-13 inhibits osteoclast formation due to downregulation of RANK. In addition, IL-4 and IL-13 was also shown to

indirectly inhibit osteoclast differentiation by binding to osteoblasts and cause a downregulation of RANKL expression (Palmqvist et al., 2006). IL-12 and IL-18 have both been shown to inhibit osteoclast formation in bone marrow cultures. This effect is, however, indirect and T cells have been indicated as a possible cell, mediating the effect through production of GM-CSF (Horwood et al., 1998; Horwood et al., 2001).

Toll-like receptors

Toll-like receptors (TLRs) are critical activators of the innate immune system. They are members of a family of receptors that share homologies with the IL-1R and are foremost expressed on antigen-presenting cells, such as macrophages and B cells. Activation of these receptors by microbial molecules, results in an amplification of inflammatory cytokines, in preparation for an adaptive immune response. TLR expression has also been detected on bone cells. TLR activation on osteoblasts induces expression of RANKL and TNF-α, and thus enhances osteoblast-mediated osteoclastogenesis (Kikuchi et al., 2001). On the other hand, activation of the TLR on osteoclast precursor cells leads to an inhibition of osteoclastogenesis (Takami et al., 2002). The reason for these opposing signals is unclear. TLR activation of osteoclasts also stimulates the production of proinflammatory cytokines, and TLRs are believed to regulate the balance between the immune system and bone metabolism, during infection of various microbes. (reviewed by Walsh et al., 2006).

The calcitonin gene family of peptides

The calcitonin family of peptides includes calcitonin (CT), two calcitonin gene-related peptides (α-CGRP, β-CGRP), amylin (AMY) adrenomedullin (ADM), intermedin/adrenomedullin2 (IMD) and three calcitonin receptor-stimulating peptides (CRSP1-3; Wimalawansa, 1997; Katafuchi et al., 2003a; Katafuchi et al., 2003b; Katafuchi et al., 2004; Roh et al., 2004; Ogoshi et al., 2004) Even though these peptides have very diverse physiological effects, they share several characteristics, important for their biological activity. In the amino terminal moiety, all peptides have a disulfide-bridged ring, very important for receptor interaction. This ring structure is followed by a potential amphipatic α-helix and a carboxy terminal amide group. Besides the amino terminal end, CT is almost entirely different from CGRP, AMY and ADM. AMY and CGRP are very similar in the amino terminal part and exhibit approximately 40% homology in the rest of the molecules. ADM exhibits 20% homology with CGRP and AMY and considerably less with CT (Wimalawansa, 1997). IMD has 33% sequence homology to ADM (Roh et al., 2004), and CRSP and CGRP have approximately 60% homology (Katafuchi et al., 2003a). Despite of these weak homologies at

the amino acid sequence level, the peptides share stronger relationships at the secondary structure level.

Calcitonin

CT was first discovered as an acute hypocalcemic hormone released from the parathyroid glands (Copp & Cheney, 1962), but shortly thereafter shown to be secreted by the thyroid C-cells (Foster et al., 1964; Zaidi et al., 2002). The hypocalcemic effect caused by CT is mainly due to its inhibitory effect on bone resorption (Friedman & Raisz, 1965), caused by the activation of calcitonin receptors (CTR) in mature osteoclasts. The effects on the osteoclast include contraction, ceased motility and decreased bone resorbing activity (Chambers et al., 1984).

Although CT can cause hypocalcemia and inhibit bone resorption, its physiological effect *in vivo* has been questioned. Thyroidectomy is a common treatment for hyperthyroidism, where the thyroid is removed and the endogenous thyroid hormones are substituted with synthetic hormones. These patients do not produce any endogenous CT, but there are no indications of any decrease in bone mass (Hurley et al.,1987). In the opposite scenario, patients with medullary thyroid carcinoma, secreting excess CT, also exhibit a normal bone structure (Hurley et al., 1987). The physiological effects on Ca^{2+} regulation have, however, been indicated in mice deficient in CT/α-CGRP, where PTH injections caused an elevated Ca^{2+} serum concentration, as compared to wild-type mice (Hoff et al., 2002). Recently, these mice have been shown to have increased bone resorption and exhibit a loss of bone mass, during lactation (Woodrow et al., 2006). These studies indicates that CT, at least under some circumstances, may have an osteoprotective role.

Mice deficient in the gene encoding CT, and the tissue-specific splice variant α–CGRP, do not have the expected decrease in bone mass due to increased bone resorption. Instead they exhibit an increased bone mass due to enhanced bone formation (Hoff et al., 2002). Since mice selectively lacking α–CGRP exhibit osteopenia caused by decreased bone formation (Schinke et al., 2004), the increased bone mass observed in CT/α–CGRP deficient mice is probably a result of the absence of CT. Similarly, heterozygous CTR deficient mice exhibit increased bone mass (Dacquin et al., 2004). However, CT/α-CGRP deficient mice exhibit an age-dependent increase of bone resorption (Huebner et al., 2006).

In addition to its well recognized inhibition of mature osteoclasts, CT has been found to inhibit PTH-stimulated multinucleated cell formation in feline marrow-derived cell cultures (Ibbotson et al., 1984) as well as in D3 stimulated multinuclear cell formation in primate marrow mononuclear cell cultures

(Roodman et al., 1985). More recently, Cornish et al. (2001) have shown that CT inhibits osteoclast formation in mouse bone marrow cultures stimulated by D3. In these studies, however, the marrow derived cells cultures are not purified and the possibility therefore may exist that CT could have exerted its effect not directly on osteoclast progenitor cells, but indirectly via contaminating cells present in the crude bone marrow cultures.

Calcitonin gene-related peptide (CGRPα/β)

CGRP is a 37-amino acid neuropeptide, identified in 1982 as a product of alternative splicing of the primary mRNA transcript of the CT gene (Amara et al., 1982; Rosenfeld et al., 1983). Alternative splicing of the mRNA leads to a tissue-specific expression of CT and CGRP; whereas CT is mostly expressed in the thyroid C-cells, CGRP is widely distributed in the nervous system and the vascular system. There are two forms of CGRP, α- and β-CGRP, encoded by two different genes. On a protein level, the two peptides only differ in one to three amino acids. CGRP is widely distributed in both the central and peripheral nervous system, mostly in sensory nerve fibers in the vicinity of the blood vessels. It is a potent vasodialator and has been suggested to be a regulator of blood flow in various organs (extensive reviews by Wimalawansa, 1997; Brain & Grant, 2004). CGRP–immunoreactive nerve fibers have also been found in bone marrow and periosteum (reviewed by Irie et al., 2002). CGRP is known to bind to osteoblasts and stimulate proliferation and has an anabolic effect on bone metabolism, since mice lacking α-CGRP exhibit an osteopenic phenotype due to decreased bone formation (Shinke et al., 2004). It is also an inhibitor of bone resorption and has been shown to inhibit the activity of mature osteoclasts (Zaidi et al., 2002), as well as the formation of multinucleated osteoclasts in D3 stimulated bone marrow cultures (Cornish et al., 2001). In these cultures, however, the target cell of CGRP action is not possible to determine.

Amylin

AMY, or islet amyloid polypeptide (IAPP), is a 37-amino acid hormone produced mainly in the pancreatic β cells. It was first identified as the major component of islet amyloid (protein deposits) of the β cells of the pancreas, in patients with type II diabetes (Cooper et al., 1987) and in amyloid deposits in tumours formed in the pancreas (Westermark et al., 1986). AMY is co-secreted with insulin after food intake, and its major physiological activity is in regulation of glucose metabolism. Its effects are opposite to that of insulin; AMY stimulates glycogen breakdown from skeletal muscles, whereas insulin promotes the production of glycogen from glucose (Wimalawansa, 1997). AMY producing cells have also been identified in the gastrointestinal tract, lung and hypothalamus, and through its actions in the central nervous system, AMY has

been shown to influence behaviour (Clementi et al., 1996) and thirst (Riediger et al., 1999). It also affects blood pressure and causes vasodilatation (Chin et al., 1994). In bone, AMY stimulates osteoblast proliferation both *in vivo* and *in vitro* (Cornish et al., 1995), and has also been shown to inhibit the activity of isolated osteoclasts as well as bone resorption in calvaria (Pietschmann et al., 1993; Cornish et al., 1994; Zaidi et al., 2002). In 2001, Cornish et al. (2001) showed that AMY could also inhibit the formation of osteoclasts in D3 stimulated bone marrow cultures and AMY deficient mice display an osteoporotic phenotype, due to increased bone resorption (Dacquin et al., 2004). The latter finding, surprisingly, indicates that AMY may be a more important physiological regulator of bone resorption than CT.

Adrenomedullin

In 1993, Kitamura et al. (1993) discovered a new peptide in human pheochromocytoma, capable of stimulating cyclic AMP (cAMP) production in platelets. Since it was expressed in the adrenal medulla as well as in pheochromocytoma which is derived from the adrenal medulla, it was called adrenomedullin (ADM) (Kitamura et al., 1993). Later on, ADM has been found in a variety of tissues such as the cardiovascular system, the central nervous system, the gastrointestinal tract, the respiratory tract and in the reproduction system. The cell types expressing ADM includes osteoblasts, fibroblasts and macrophages. The effects caused by ADM are very diverse and it exerts its effects both as a circulating hormone and as a local paracrine mediator (extensively reviewed by Hinson et al., 2000; Beltowski & Jamroz, 2004). Human ADM consists of 52 amino acids. It is first produced as a prepro-peptide of 155 amino acids. Cleavage of a 21 amino acid signalling sequence in the amino terminal, converts the peptide to pro-ADM, the precursor of ADM and another related peptide, pro-adrenomedullin N-terminal peptide (PAMP). ADM has been shown to stimulate proliferation in primary osteoblasts and osteoblast-like cells (Cornish et al., 1997). Using neonatal murine calvarial cultures, ADM has also been shown to stimulate thymdine incorporation, indicating a stimulatory effect on bone formation (Cornish et al., 1997).

Calcitonin receptor-stimulating peptide (CRSP)

CRSP was discovered in 2003 as a new member of the CT family of peptides (Katafuchi et al., 2003a). It was identified from a porcine brain extracts, in search for the endogenous ligand of the calcitonin receptor expressed in the central nervous system. CRSP is a 38-amino acid peptide and in resemblance to the other members of the CT family, CRSP has a terminal amide group in the carboxy terminal as well as the characteristic ring structure in the amino terminal,

between two cysteins residues in position 2 and 7 (Katafuchi et al., 2003a). Porcine CRSP show the highest amino acid sequence homology with human and porcine CGRP (60%), but unlike CGRP, CRSP does not affect blood pressure. Searches in databases of the porcine hypothalamus cDNA led to the identification of two additional CRSPs (designated CRSP-2 and 3) but these peptides have not been shown to have any physiological effects. To date, CRSP has been found in porcine, bovine and canine cDNA libraries, but so far, no human or rodent counterparts have been identified (Katafuchi & Minamino, 2004). Analyses of CRSP mRNA and protein show that the highest expression of this peptide is found in the midbrain, hypothalamus and the thyroid gland, but it is also detected in the cerebral cortex, thalamus and the pituitary. Administration of CRSP to rat decreased serum calcium (Hamano et al., 2005). This effect may be a result of activation of the CTR in osteoclasts since CRSP-1 decreases osteoclast formation in 1,25$(OH)_2$-vitamin D3 stimulated co-cultures of spleen cells and stromal cells, as well as in M-CSF and RANKL stimulated bone marrow cells (Notoya et al., 2007). It has also been shown that CRSP, similar to CT, stimulate cAMP formation, inhibit proliferation and reduced Ca^{2+} uptake in the renal epithelial cell line LLC-PK1 (Hamano et al., 2005). These data indicate that CRSP is a systemic regulator of serum calcium concentrations, by a mechanism similar to that of CT.

Intermedin /Adrenomedullin 2

IMD (also known as ADM2) was first described as a hormone produced in the intermediate lobe of the pituitary gland (Abramowitz et al., 1943). It was later identified as a homologue to CGRP and ADM (Ogoshi et al., 2003; Roh et al., 2004) and considered to be a new member of the CT family of peptides. IMD is produced as a prepro-hormone of 148 amino acids, and thereafter processed into a 47-amino acid peptide called IMD-long. IMD can also be further processed into a 40-amino acid peptide, IMD-short (Roh et al., 2004). In resemblance to the other members of the CT family, IMD has an amidated carboxy terminal and a ring structure between two conserved cysteine residues in the amino terminal. Similar to ADM and CGRP, IMD has been shown to affect blood pressure and heart rate (Pan et al., 2005; Ren et al., 2006; Taylor et al., 2005a). IMD also inhibits food and water intake and suppress gastric emptying (Fujisawa et al., 2004; Roh et al., 2004; Taylor et al., 2005a). In addition, IMD has been shown to stimulate the release of prolactin from the pituitary gland (Chang et al., 2005; Taylor et al., 2005b), as well as stimulate hypothalamic oxytocin-secreting neurons (Hashimoto et al., 2005). It is currently not known if IMD has any effect on bone cells.

Receptors for the calcitonin gene family of peptides
G protein-coupled receptors

The G protein-coupled receptor family (GPCRs) is one of the largest and most diverse receptor families. The members have been divided into six classes, based on their sequence homologies and functional similarities. These receptors have in common that they are coupled to a trimeric guanine-binding protein (G protein) composed of three different polypeptide chains; the α-, β- and γ-chain. When the G protein is inactive, the β- and γ-chain form a tight complex which anchors the G protein to the membrane, whereas Gα binds GDP and is coupled to the GPCR. When the receptor, and subsequently the G protein is activated, GDP bound to the Gα subunit is exchanged for GTP. This induces a conformational change in the G protein, which dissociates and the subunits are free to act upon their effectors and thereby relay the intracellular signal (McGarrigle & Huang, 2007; Kroeze et al., 2003).

The group B family of the GPCRs consists of large receptors, characterized by having a heptahelical region, i.e., seven transmembrane regions. Some of the intracellular signalling pathways activated by GPCRs are adenylate cyclase/cAMP, adenylyl phosphokinase C/Ca^{2+} and the phospholipase C/phosphoinositide cascade. In human, there are at least 18 different Gα chains (Hermans, 2003; Wong, 2003), five Gγ chains and 11 Gβ chains (Hermans, 2003) to which GPCRs can bind. Recently, it has been suggested that GPRCs can interact directly, with effectors molecules other than the trimeric G protein as well (McGarrigle & Huang, 2007).

The calcitonin receptor

The CTR was identified in 1991 (Lin et al., 1991) and belongs to the group B family of GPCRs which also includes the parathyroid hormone/parathyroid hormone related peptide (PTH-PTHrP) receptor, and receptors for secretin, vasoactive intestinal peptide (VIP), growth hormone releasing hormone (GHRH), and glucagone-like peptide 1 (Lin et al., 1991; Goldring et al., 1993). Due to alternative splicing of the mRNA transcript there are several isoforms of the CTR. In rodents, there are two isoforms of the CTR, designated C1a and C1b, which differ in a 37-amino acid insert in the second extracellular domain of C1b. The significance of this insert is not fully understood but C1a predominates in mouse and rat osteoclasts although both forms are expressed (reviewed by Pondel, 2000; Findley & Sexton, 2004). The downstream signalling of CTR has been linked to both adenylate cyclase/cAMP-protein kinase A and to protein kinase C/Ca^{2+} (Purdue et al., 2002). The adenylate cyclase-coupled CTR signalling is summarized in fig. 3. In addition, activation of the CTR has also been shown to stimulate the phosphorylation of the MAP kinase ERK1/2 in

HEK293 stably expressing the rabbit CTR C1a (Chen et al., 1998) as well as in rabbit and murine osteoclasts (Zhang et al., 2002).

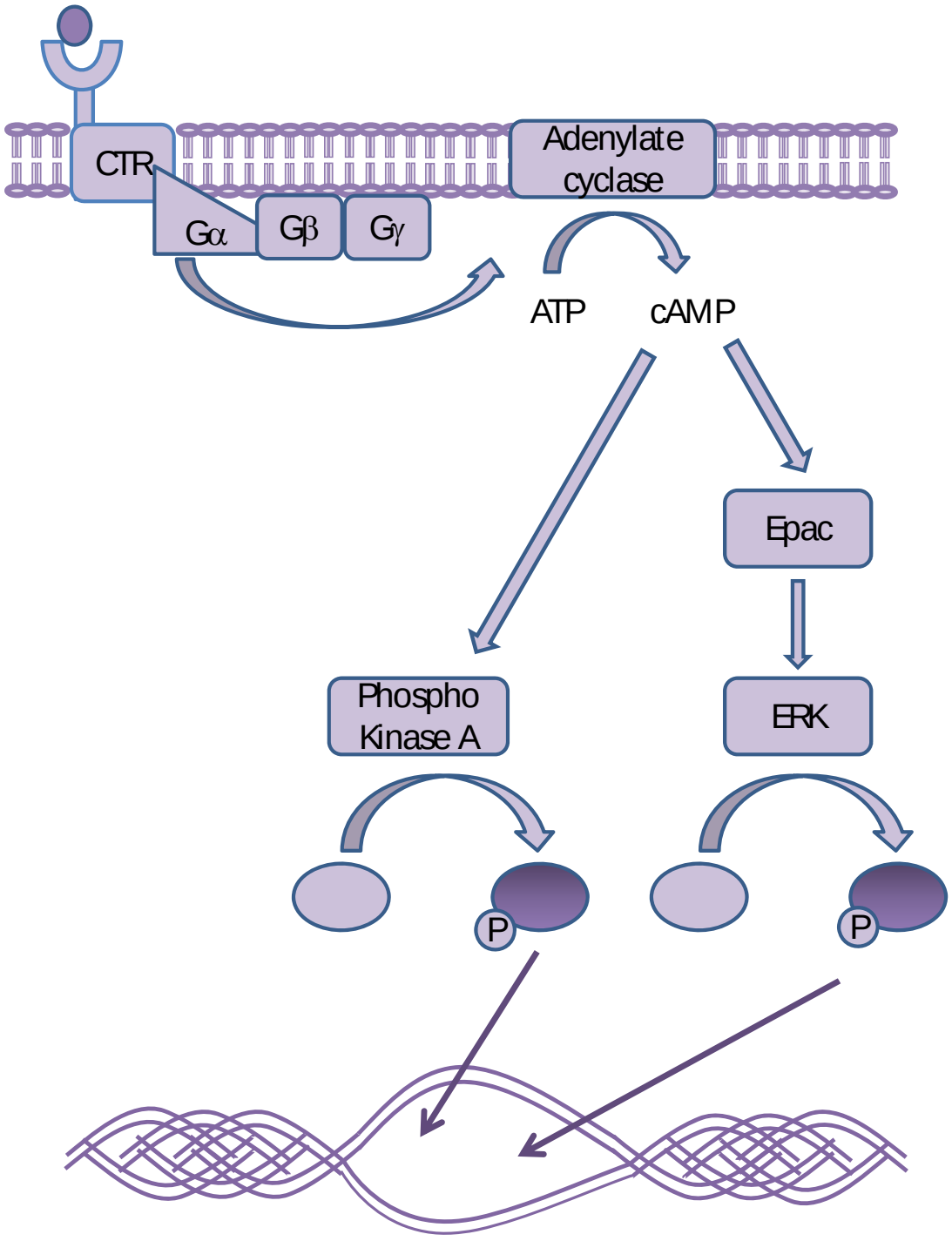

Figure 3. CTR signalling via adenylate cyclase. Activation of adenylate cyclase stimulates cAMP production. cAMP in turns activates several signaling pathways leading to an increased kinase activity resulting in phosphorylation and hence activation of transcription factors and subsequent transcription of target genes.

The CTR is expressed in several tissues, including cells in the central nervous system and epithelial cells of the kidney but is, however, most associated with the expression on mature osteoclasts (Nicholas et al., 1986). The CTR is not expressed on the very early osteoclast progenitor cells but is induced during osteoclast differentiation (Lee et al., 1995; Quinn et al., 1999).

The calcitonin receptor-like receptor

The calcitonin receptor-like receptor, denoted CRLR or CRL, was first discovered as an orphan receptor with large sequence homologies to the CTR (55% homology). The CRLR is a unique member of the GPCR family of receptors, in the sense that it requires an accessory protein for expression and function. The CRLR was suspected to be the receptor for CGRP and in 1993, Njuki et al. (1993) transfected the CRLR into COS-7 cells and stimulated the cells with CGRP but did not detect any response. In 1996, Aiyar et al. (1996) showed that HEK293 cells, transfected with CRLR cDNA, could respond to CGRP with a 60-fold increase of cAMP production due to activation of adenylyl cyclase. The explanation to the discrepancy between these results was found in 1998 when McLatchie et al. (1998) discovered a 148-amino acid protein, denoted receptor activity-modifying protein 1 (RAMP1). They were able to show that the functional receptor for CGRP was a complex formed by the CRLR and RAMP1. Unlike COS-7 cells, HEK293 cells express endogenous RAMP1 and could therefore respond to CGRP. Searches in databases led to the discovery of two additional RAMP1-like proteins; RAMP2 and RAMP3 (McLatchie et al., 1998).

Receptor activity modifying protein 1-3

The RAMP proteins have an extracellular amino terminal domain, a short intracellular carboxy terminal domain and a single-transmembrane spanning α-helix. The RAMP proteins share about 30% sequence identity and in the amino terminal there are four highly conserved cysteine residues (McLatchie et al., 1998; reviewed by Udawela et al., 2004; Hay et al., 2006; Sexton et al., 2006). The amino terminal moiety has been indicated to be involved in ligand recognition (Udawela et al., 2006), the transmembrane region seems important for forming a stable complex with the receptor (Steiner et al., 2002), whereas the carboxy terminal domain may influence intracellular signalling upon ligand binding (Udawela et al., 2006). The functions of the different domains are, however, not fully known. Unlike RAMP2 and RAMP3, RAMP1 cannot be translocated to the cell surface unless it forms a complex with a receptor. Instead, it remains in the ER in as homodimers, formed by intermolecular disulphide bonds.

Heterodimerization of CRLR with one of the three RAMPs is necessary for translocation of the receptor complex to the cell surface. Upon heterodimerization, the receptor is terminally modified and glycosylated. However, it is not clear if this glycosylation is necessary for full activity of the receptor. When expressing CRLR and the RAMP proteins in insect cells, where no glycosylation occurs, fully functional receptors are still formed (Hay et al., 2006; Sexton et al., 2006). In addition to function as a chaperon for CRLR and alter ligand specificity, the RAMP proteins have also been implicated to influence the receptor complex compartmentalization and the intracellular signalling following receptor activation. Christopoulos et al. (2003) have reported that co-expression of the VIP/PACAP receptor 1 (VPAC1) with RAMP2, in COS-7 cells, significantly enhanced the phosphoinositide signalling in response to VIP stimulation. In contrast, RAMP2 expression did not affect the accumulation of cAMP after VIP treatment. More recently, the RAMP proteins have also been shown to influence post-endocytotic receptor recycling/degradation pathways (Bomberger et al., 2005; Cottrell et al., 2007).

Receptor component protein

The CRLR receptor complex consists of another component as well. Receptor component protein (RCP) is a 148-amino acid intracellular protein that is required for efficient signalling of CGRP and ADM via the CRLR receptor complex. In cells expressing RCP antisense constructs, the responsiveness to CGRP and ADM was reduced whereas no effects on ligand affinity or receptor density were seen. It has therefore been suggested that the role of RCP is to couple the receptor to the intracellular signalling pathways (Evans et al., 2000; Prado et al., 2002).

Receptor complexes formed by the CTR, the CRLR and the three RAMPs

All three RAMPs can form functional complexes with either CTR or CRLR, and the different combinations determine ligand recognition. McLatchie et al. (1998) showed that CRLR in combination with RAMP1 functioned as a receptor for CGRP, whereas CRLR in combination with RAMP2 responded to ADM but not to CGRP. The general view is that the CTR in combination with one of the three RAMP proteins functions as a receptor for amylin, the CRLR in combination with RAMP1 functions as a receptor for CGRP, whereas CRLR and RAMP2 or RAMP3 recognizes ADM (Poyner et al., 2002; Hay et al., 2006; Sexton et al., 2006). In addition, the CTR alone functions as a receptor for either CT or CRSP, and IMD seem to be recognized by both CTR and CRLR in combination with

one of the RAMP proteins (Roh et al., 2004, Takei et al., 2004). The different receptor complexes are summarized in Table 1.

Table 1. Receptors for the calcitonin gene family of peptides.

Receptor	Receptor components	Ligand
ADM1	CRLR+RAMP2	Adrenomdeullin
ADM2	CRLR + RAMP3	Adrenomedullin
AMY1	CTR + RAMP1	Amylin
AMY2	CTR + RAMP2	Amylin
AMY3	CTR + RAMP3	Amylin
CGRP	CRLR + RAMP1	CGRP
CRSP	CTR	CRSP
CT	CTR	Calcitonin
IMD	CT/CRLR + RAMP1-3	Intermedin

Expression and distribution in tissues

RAMP proteins are expressed throughout the body and the distribution of RAMPs overlap, but are not consistent with expressions of CTR and CRLR. Therefore, it was suggested that RAMP proteins may interact with other receptors as well. In fact, several other members of the GPCR superfamily have been shown to interact with at least one of the three RAMPs (Christopoulus et al., 2003; Reviewed by Sexton et al., 2006). Recently, RAMP2 and RAMP3 have also been shown to interact with the calcium-sensing receptor (CaSR), which belongs to another class of the GPCRs (Bouschet et al., 2005).

CT, CGRP, AMY, ADM, and CRSP have all been implicated to affect bone metabolism, although the mechanisms are not fully understood. The receptors, recognizing the peptides of the CT family seem to be somewhat promiscuous, making it harder to evaluate the effect of one peptide *in vivo*, as it may be compensated for by another.

The discovery of the RAMP proteins (McLatchie et al., 1998), having the capacity of altering the specificity of the receptor for different ligands, may be an important part in the explanation to how different cell types can alter their susceptibility to different effector substances. In development of new treatments of pathological conditions caused by impaired bone metabolism, RAMPs may be a strong candidate as a new target. Analyses of a CGRP antagonist, BIBN4096BS, have shown that the receptors preference between CGRP and the antagonist is dependent on one single amino acid residue in the amino terminal of RAMP1 (Dood et al., 2000; Mallee et al., 2002). ADM receptors, consisting of CRLR in combination with RAMP2 or RAMP3 are not antagonized by BIBN4096BS (Hay et al., 2003; Salvatore et al., 2004), further indicating that

RAMP1 is required for recognition of this antagonist. Finding such specific target residues within the receptor or RAMP proteins could be very useful in development of new, possibly cell specific, therapeutic drugs.

There are very few studies on the expression and regulation of CRLR and the RAMP proteins in bone cells. Nakamura et al. (2005) have reported, using laser micro dissection, that multinucleated osteoclast-like cells from co-cultures of mouse spleen and bone marrow cells, express mRNA for CTR, CRLR and RAMP2, but not for RAMP-1 and -3. Osteoblasts-like cells also express CRLR and all three RAMP proteins (Shinke et al., 2004; Uzan et al., 2004). Uzan et al. (2004) reported that treatment of dexomethasone, a synthetic glucocorticoid, caused a downregulation of the CRLR expression at mRNA as well as protein level, an upregulation of RAMP-1 and -2 mRNA, whereas RAMP3 mRNA was unaffected. The effects of the CT family of peptides on expression of the receptor components in bone cells are, however, completely unknown.

AIMS

The overall aims of this thesis were to evaluate the presence, function and regulation of receptors for the peptides of the CT family in osteoclasts and osteoblasts and if these peptides caused effect on receptor expression or differentiation of bone cells.

The first aim was to examine the presence of the calcitonin receptor, the calcitonin receptor-like receptor and the receptor activity-modifying proteins on differentiating osteoclast progenitor cells, i.e. in spleen cells and bone marrow macrophages. In addition, we aimed to examine whether these receptor components could form functional receptors, responsive to the members of the calcitonin gene family of peptides, and if activation of the functional receptors affected the regulation of the expression of the receptor components. (Paper I & II)

Next, we sought to examine the effects of the peptides on osteoclast differentiation, and once their inhibitory effects were observed we aimed to analyze the mechanism of action. (Paper I & III)

Very little is known about the effects of CRSP and IMD on bone resorption and, therefore, one aim has been to study the effects of these peptides in more detail. (Paper III)

Finally, one aim has been to examine the presence of the receptor components and their function, on osteoblastic cells at different levels of differentiation. (Paper IV)

METHODS

CsA mice from our own inbred colony were used in all experiments. Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

Isolation and culture of primary mouse spleen cell cultures

The spleens of 5-to-9-week-old mice were dissected free of adhering tissues and cells were released by rubbing the spleens against the bottom of a Petri dish, in which grooves had been made by a scalpel. Erythrocytes were lysed in red blood cell lysis buffer (0.16 M NH_4Cl, 0.17 M Tris, pH 7.65) and the remaining cells were seeded, at a cell density of 10^6 cells/cm^2. The cells were cultured in α-MEM supplemented with 10% FBS, L-glutamine (0.7 mM), 100 U/ml bensylpenicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamycin sulphate. Cells were allowed to settle over night in complete medium and thereafter the medium was changed and the experiment started. Osteoclast precursor cells were induced to proliferate and differentiate by the addition of M-CSF (25 ng/ml) and RANKL (100 ng/ml).

To study osteoclastogenesis, cells were grown in medium containing 25 ng/ml of M-CSF + 100 ng/ml of RANKL, with or without test substances. Cells cultured in complete medium without M-CSF and RANKL were included in all experiments as a control. All cells were maintained at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Medium was changed after 3 d. After 1-6 d, the cells were washed three times with PBS (pH 7.35) and fixed with acetone in citrate buffer/3% formaldehyde. Cells were stained for TRAP activity using an Acid Phosphatase Leukocyte staining kit and by following the manufacturer´s instruction. Multinucleated (no of nuclei ≥3), TRAP positive cells were counted as osteoclasts. Osteoclasts formed were able to make resorption pits when spleen cells were cultured on bovine bone slices. No osteoclasts were formed when cells were treated with either M-CSF or RANKL alone and the stimulation caused by M-CSF and RANKL was abolished by osteoprotegerin (OPG) (data not shown). No osteoclasts were formed in the presence of PTH or D3 (data not shown), indicating the lack of stromal cells in the spleen cell cultures.

Isolation and culture of primary mouse bone marrow macrophages

The femurs and tibiae from 5-to-7-weeks-old male mice were dissected out and cleaned from adhering tissues. The cartilage ends were cut off and the cells in the marrow cavity were flushed out by α-MEM in a syringe with a sterile needle. The marrow cells were collected in α-MEM/10% FBS and the erythrocytes lysed

(see spleen cell cultures above). The remaining bone marrow cells were washed and suspended in α-MEM/10% FBS containing L-glutamine (0.7 mM), antibiotics (100 U/ml bensylpenicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamycin) and 100 ng/ml M-CSF. The cells were seeded at a density of 8 x 10^4 cells/cm^2 in a 60 cm^2 culture dish, to which stromal cells and lymphoid cells cannot adhere. After 3 d, the cultures were vigorously washed with PBS twice and the cells attached to the bottom were then detached using 0.02% EDTA in PBS. These cells were resuspended in α-MEM/10% FBS with 100 ng/ml M-CSF and then seeded at a density of 0.5 x 10^4 cells/cm^2 in 60 cm^2 dishes. After another 3 d, the cells were washed and detached as described for the initial 3 d culture period and used as bone marrow macrophages (BMM). For further details, see Takeshita et al. (2000). These cells did not express alkaline phosphatase, RANKL, OPG or CTR mRNA, but mRNA for RANK, c-Fms, cathepsin K and TRAP, as assessed by quantitative real-time PCR (data not shown).

For osteoclastogenesis experiments, BMM were seeded at a density of 10^4 cells/cm^2 in α-MEM/10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 100 ng/ml of RANKL, with or without test substances. Medium was changed after 3 d. After 4-5 d, the cultures were harvested and the cells fixed with acetone in citrate buffer/3% formaldehyde and subsequently stained for TRAP. The TRAP positive cells with three or more nuclei were considered osteoclasts and the number of multinucleated osteoclasts was counted. In another set of experiments, non-adherent bone marrow cells were isolated from femurs of CsA mice as described elsewhere (Roach et al., 1997). In this method, cells isolated from bone marrow are allowed to attach to culture plates for 2 h. Thereafter, the cells that have not attached are collected, resuspended in α-MEM with 10% FBS, L-glutamine, and antibiotics and seeded at 10^6 cells/cm^2. After incubation with M-CSF (25 ng/ml) and RANKL (100 ng/ml), with or without test substances, for 4 d, the cells were fixed and stained for TRAP and the number of TRAP positive multinucleated osteoclasts counted.

Analysis of osteoclast activity by measurement of ^{45}Ca release from pre-labelled neonatal calvarial bones

Neonatal mice were injected with 1.5 µCi ^{45}Ca 4 d prior to dissection to label the mineral part of the skeleton. At the age of 6-7 d, calvarial bones were microdissected and cultured as described elsewhere (Lerner, 1987; Ljunggren et al., 1991). The bones were preincubated for 1 d in α-MEM containing 0.1% albumin and 1 µM indomethacin. In order to prestimulate osteoclast formation and bone resorption before the experiment started, bones were precultured for another 1 d with PTH or 1,25$(OH)_2$-vitamin D3. One group of bones were preincubated and then further cultured in the absence of stimulator as an unstimulated control group. Following the incubation period in PTH or D3,

bones were cultured in the presence of PTH or D3 with or without test substances for 1 d. To accumulate data from several experiments a 100-%-transformation was made for each experiment; the ^{45}Ca-release in the PTH groups was referred to as maximum release and considered 100%. In the time-course experiments, the skeleton was pre-labelled by injecting 12.5 μCi ^{45}Ca and the bones were initially precultured, and then prestimulated with PTH and subsequently cultured for 3 d in the presence of PTH with or without test substances as described above. The kinetics of the release of ^{45}Ca was analysed by the withdrawal of small amounts of medium at the stated time points.

Analysis of osteoclast activity by measurement of collagen degradation

Calvarial bones of 6-to-7-day-old mice were also used to analyse bone resorption by analysing extracellular matrix breakdown, as assessed by the amount of collagen degradation fragments in culture media. Calvarial bones were microdissected and cultured as described above. Following the two preincubation periods, collagen fragments in culture media were quantified using a commercially available RatLaps ELISA kit, following the manufacturer's instructions.

Isolation and culture of primary mouse calvarial osteoblasts

Bone cells were isolated from calvariae of 2-to-3-day-old CsA mice using bacterial collagenase using the modified time sequential enzyme-digestion technique described by Boonekamp et al., 1984. Cells from populations 6–10, showing an osteoblastic phenotype as assessed by their cAMP-responsiveness to parathyroid hormone (PTH), expression of alkaline phosphatase, osteocalcin, and bone sialoprotein expression, as well as the capacity to form mineralized bone nodule, in the presence of ascorbic acid and β-glycerophosphate (data not shown), were used. The cells were seeded in culture flasks containing α-MEM supplemented with 10% FBS, l-glutamine, and antibiotics at 37 °C in humidified air containing 5% CO_2. After 4 d, the cells were seeded at a density of 5 000 cells/cm^2. Cells were cultured in complete medium with or without bone morphogenic protein2 (BMP-2; 250 ng/ml), for 1-18 d before used for RNA extraction or cAMP analyses.

Culture of the non-transformed murine osteoblastic cell line MC3T3-E1

Osteoblastic MC3T3-E1 cells were plated at 5 000 cells/cm^2 in 4 cm^2 wells overnight in α-MEM supplemented with 10% FBS, antibiotics and glutamine, before change to fresh medium with or without BMP-2 (250 ng/ml). Cells were harvested at d6 and d12 and RNA extracted. The medium was changed every 3 d.

RNA isolation and first strand cDNA synthesis

Spleen, BMM cells, mouse calvarial osteoblasts and MC3T3-E1 cells were isolated, and cultured as described above. At d1-18, the cells were collected using a cell scraper and total RNA was extracted. Total RNA was extracted using either TRIzol reagent or by using the RNAqueous^{TM}–4PCR kit according to the manufacturer's instructions. Samples were subsequently digested with DNAse. The quality of the RNA preparations was analysed in 1.5% agarose gel electrophoreses and visualised using ethidium bromide. Single-stranded cDNA were synthesised from 0.1-1.0 μg of total RNA using a 1st strand cDNA synthesis kit with avian myeloblastosis virus (AMV) and oligo$(dT)_{15}$ primers, according to the manufacturer's protocol. To ensure that there was no genomic DNA in the samples, reactions without AMV reverse transcriptase was included as a negative control. The mRNA expression was then analysed, using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), or quantitative real-time PCR.

Semi-quantitative reverse transcriptase-polymerase chain reaction

First strand cDNA was amplified by PCR using a PCR core kit and PC690G Gradient Thermal Cycler (Corbett Research, Australia) or Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR reactions were performed using PCR standard protocol. The conditions for PCR were: denaturing at 94^{o}C for 2 min, annealing at various temperatures for 35s, followed by elongation at 72^{o}C for 40 s; in subsequent cycles denaturing was performed at 94^{o}C for 35s. Annealing temperatures were optimized for each individual primer pair. Some PCR reactions were initiated with hot start at 95^{o}C for 15 min , using a HotStar Taq polymerase kit. Some PCR reactions were performed with a step down technology, where the primer annealing temperature was 65^{o}C for the first ten cycles, and then decreased by 5^{o}C every 5 cycles down to 45^{o}C. The expressions of these factors were compared at the logarithmic phase of the PCR reaction, the products separated in electrophoreses on a 1.5% agarose gel, and visualised using ethidium bromide. Primers were designed using ABI Prism^{TM} Primer express^{TM} (Applied Biosystems, Foster City, CA, USA) or Omiga^{TM} (Genetic Computer

Group Inc, Madison, WI, USA). The identity of the PCR products was confirmed using a QIAquick purification kit and a Thermo Sequence-TM II DYEnamic ET^{TM} terminator cycle sequencing kit with sequences analysed on an ABI377 XL DNA sequencer.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR analyses were performed using the TaqMan Universal PCR master mix or Sybr Green PCR master mix and a sequence detection system (ABI Prism 7900 HT Sequence Detection System and Software, Applied Biosystems, Foster City, CA, USA). PCR analyses were performed using TaqMan® gene expression assays or primers and fluorescence labeled probes (reporter fluorescent dye VIC at the 5' end and quencher fluorescent dye TAMRA at the 3' end) designed using ABI Prism^{TM} Primer express^{TM} (Applied Biosystems). To control variability in amplification due to differences in starting mRNA concentrations, β−Actin was used as an internal standard. The relative expression of target mRNA was computed from the target Ct values and the β−Actin Ct value using the standard curve method (*User Bulletin #2,* Applied Biosystems).

Immunocytochemistry

BMM cells were isolated and cultured as described above. At d1-4, cultures were washed in PBS, air dried in room temperature and thereafter fixed with cold acetone (-20^{o}C) for 20 min. Unspecific binding was blocked with PBS/4%BSA and the cultures were thereafter incubated with antibodies recognizing mouse CTR. The anti-CTR antibody was a kind gift from Dr P. Sexton, Monash University, Australia. After a washing step, the cultures were incubated with a secondary antibody (swine-anti rabbit IgG) conjugated to FITC. Controls included cells incubated with the appropriate isotype controls, as well as cells incubated with only the secondary antibody. The cells were then analysed using a Leica DMRBE microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) together with a Leica DC200 digital camera and Leica DC200 software (Leica Microsystems AG, Wetzlar, Germany). Another set of cultures were fixed with acetone in citrate buffer and subsequently stained for TRAP.

Western blot

BMM cells isolated as described above were seeded at a density of 2 x 10^4/cm^2 in 60 cm^2 culture dishes. Cells were harvested at d2 and washed three times with ice-cold PBS (pH 7.2). The cells were suspended in 0.6 ml RIPA buffer (1%

Igepal CA-630, 0.1% SDS, 2 mM EDTA, 50mM NaF, in PBS) containing protease inhibitors (0.1 mg/ml PMSF, 1 mM natriumorthvanadat, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) and incubated at 4°C for 15 min. The cells lysates were transferred to 1.5 ml tubes and incubated on ice for an additional 60 min and thereafter centrifuged (10 000 *g* at 4°C, 5 min) The protein concentration was determined using a BCA protein assay kit (Pierce) with bovine albumin as standard. If necessary, the samples were concentrated using centrifugal filter devices, according to manufacturers' instructions. The proteins were separated in SDS-polyacrylamid gel electrophoresis (7.5% polyacrylamid for CTR and CRLR and 12% for RAMP1, RAMP2, RAMP3 and actin detection), thereafter blotted onto a nitrocellulose filter. The membranes were incubated in blocking solution of TBS (150 mM NaCl, 20 mM Tris, pH 7.2) with 1% dry-milk and 1% BSA for 60 min in room temperature before incubation with the primary antibody over night in 4°C. The primary antibody was diluted 1:1000 in TBST (150 mM NaCl, 20mM Tris, 0.05% Tween, pH 7.2) with 1% dry-milk and 1% BSA. The membranes were washed three times in TBST to remove unbound antibodies and then incubated with the secondary, peroxidase-linked antibody for 60 minutes in room temperature. After washing three times in TBST, proteins were detected using an chemiluminescense kit according to manufaturer's instructions and detected using Chemi Doc™ XRS (BIORAD Laboratories AB, Sundbyberg, Sweden)

Fluorescence-activated cell sorting (FACS)

Crude bone marrow cells and BMM cells, obtained as described above, were washed with PBS/3% FBS and stained with antibodies (0.4 μg/10^6 cells) against the macrophage markers mouse CD11b and CD115, or the lymphoid cell markers CD3 and CD45R. 10 000 cells were analyzed from each sample. Debris and cell fragments were excluded by a threshold value of approximately 50% of the FSC of the mean population FSC. The cells were analysed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA).

Cell sorting

BMM cells, obtained as described above, were washed with PBS/3% FCS and stained with antibodies (0.4 μg/10^6 cells) in PBS/3% FCS against the mouse macrophage marker CD115 (c-Fms), and the mouse lymphoid cell markers CD3 (T cell marker) and CD45R (B cell marker). $CD115^+$ $CD3^-$ $CD45R^-$ cells were sorted by flow cytometry in the flow cytometer cell sorter FACSVantageDiVa (BD, Biosciences) and seeded at a density of $10^4/cm^2$ in α-MEM/10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 100 ng/ml of RANKL, with or without test substances. After 4-5 d, with a change of

medium at d3, the cultures were harvested and the cells fixed with acetone in citrate buffer and subsequently stained for TRAP.

MTT based cell growth determination

BMM cells were seeded at a density of $10^4/cm^2$ in 96-well plates in medium containing M-CSF (100 ng/ml) +/- RANKL (100 ng/ml) +/- CRSP or IMD and incubated for 1-4d. Cells were harvested and analysed using a commercially available 3-[4,5-dimetylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide (MTT) based cell growth determination kit.

TRAP activity assay

Spleen cells were isolated and cultured, as described above. After 4 d, the cells were washed in PBS and lysed in Triton X-100 (0.2% in H_2O). After centrifugation, supernatant was collected and kept at -20^oC until analyses. TRAP activity was determined using p-nitrophenyl phosphate as substrate at pH 4.9, in the presence of tartrate (0.17 M). The activity of the enzyme was assessed as the OD_{405} of liberated p-nitrophenol, and normalized to the amount of cell protein analysed using a BCA protein assay kit. The enzyme assays were performed under conditions where the reaction was proportional to amount of enzyme and reaction time.

Cyclic AMP formation

BMM, primary osteoblast-like cells and calvarial bones were isolated and cultured as described above. After 2-4 d (BMM), 2-12d (osteoblastic cells), and 1 d (calvarial bones), cells were washed in serum-free α-MEM, and thereafter incubated in HEPES-buffered α-MEM containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) for 30 min, after which test substances were added. After 5 min (cells) or 10 min (calvaria), cultures were harvested and cAMP was extracted from the cells, using 90 % n-propanol. Propanol was removed by evaporation and the remaining sample was resuspended in assay buffer and analyzed using a commercially available cAMP [^{125}I] Radioimmunoassay Kit, according to manufacturers' instructions.

Pit formation

Bone slices with a diameter of 5 mm and a thickness of 100 μm were prepared from bovine femur, washed in ethanol and PBS and then ultrasonicated. The

slices were placed at the bottom of 96-well plates and BMM (3 200 cells/well) were added in 125 µl α–MEM/10%FBS with 100 ng/ml M-CSF and 100 ng/ml RANKL. Cells were incubated with or without test substances, for 7 d, after which the cells were removed with trypsin and ultrasonication and the bone slices stained with toluidine blue to detect the resorption pits.

Cells were also seeded (3 200 cells/well) in Biocoat osteologic disks, coated with a calcium phosphate film, for 7 d. Cells were thereafter removed and the unstained slides examined using light microscopy.

Apoptosis

Spleen cells were isolated and cultured as described above. After 3d, CT was added to the cultures, which were then incubated for 4 h before harvest. Cultures without CT were used as a control. Cells were washed in PBS and lysed. DNA fragmentation was then analysed using Cell Death Detection ELISA (Roche).

Statistical analyses

All statistical analyses were performed using one-way analysis of variance (ANOVA) with Levene's homogenicity test, and post-hoc Bonferroni's, or where appropriate, Dunnett's T3 test or using the Independent-Sample T test (SPSS for Windows, Apache Software Foundation). All experiments have been performed at least twice with comparable results and all data are presented as the means ± SEM.

RESULTS & DISCUSSION

Receptor expression during osteoclast differentiation

For studies of the receptor expression in osteoclasts we used BMM cultures. The advantage with BMM cultures is the high degree of purity compared to spleen cell cultures. The BMM we use are devoid of lymphocytes, as assessed by the lack of cells expressing CD3 or B220. All cells express CD11b/Mac1 and about 75% express CD115/c-Fms, reflecting the monocyte/macrophage phenotype. These cells do not express ALP, RANKL, OPG or CTR mRNA, as assessed by quantitative real-time PCR. In addition, stimulation by PTH or D3 cannot induce osteoclastogenesis. This indicates the absence of stromal cells in these cultures.

We found that mRNA for CRLR and RAMP1-3 were expressed in M-CSF stimulated cultures. CTR was not expressed, but addition of RANKL caused an upregulation of this mRNA. This was expected since RANKL induce osteoclast differentiation and the CTR is a hallmark of mature osteoclasts. When comparing the mRNA levels to that of brain (not shown), we found that RAMP2 and RAMP3 were expressed at very low levels in preosteoclasts, whereas CTR, CRLR and RAMP1 were expressed at clearly detectible levels. Because of the low levels of RAMP2 and RAMP3, we excluded them from further mRNA analyses. When following the expression during the differentiation phase (d1-4) we could see that RANKL caused a transient upregulation of CRLR mRNA expression at d2, and that RAMP1 mRNA expression was transiently downregulated at the same time point.

Western blot analyses of CRLR protein in BMM, cultured for 2 d in M-CSF, revealed two fragments (74 kDa, 60 kDa). These fragments assumably correspond to different glycosylated forms of the CRLR. We also detected fragments corresponding to the RAMP1 homodimer (37 kDa). RAMP1 form homodimers, in the absence of a receptor and is retained in the ER in this form. This homodimeric complex is very stable and maintained even under reducing conditions (Sexton et al., 2001). In the RAMP2 analyses, we detected a dense fragment at approximately 37 kDa. According to the estimated size of RAMP2 of 20 kDa this could correspond to a RAMP2 homodimer, as seen in the RAMP1 analyses. Unlike RAMP1, RAMP2 and RAMP3 contain multiple glycosylation sites, and this could also affect the size of the protein. Western blot analyses of RAMP3 revealed a dense band at 37 kDa and weaker band at 27 kDa. The 27 kDa fragment presumably represents a glycosylated form of RAMP3.
FACS analyses of total, as well as membrane bound protein, showed that CRLR as well as all three RAMP proteins could be detected on BMM cells. However, since the Western blot analyses of RAMP3 revealed an unidentified fragment of 37 kDa, the FACS analyses of RAMP3 should be considered with caution.

RANKL did not affect any of the protein levels, as assessed by FACS or Western blot. Immunocytochmical analyses of the CTR expression showed that in non-permeabilized BMM no CTR-like antigen was expressed. Addition of RANKL to the cultures induced the CTR protein expression which could be detected on mononucleated cells at d2-3. After 4d, both mono- and multinulceated cells expressed the CTR antigen on the cell surface.

Nakamura et al. (2005) have reported, using laser capture microdissection (LCM), that D3 stimulated multinucleated osteoclast-like cells derived from co-cultures of mouse spleen and bone marrow cells, express mRNA for CTR, CLR and RAMP2, but not for RAMP-1 and -3. Our findings show, for the first time, that osteoclast progenitor cells express CTR, CRLR, RAMP2 as well as RAMP-1 and -3 and therefore express the components needed to form receptors for CGRP, ADM and IMD. The fact that we could detect all three RAMPs in 4d-cultures of M-CSF/RANKL stimulated BMM cells, whereas Nakamura et al. (2003) only could find RAMP2 in mature osteoclasts, could be due to that osteoclast progenitor cells express all three RAMPs but that RAMP1 and RAMP3 are downregulated when these cells become multinucleated. This possibility cannot be excluded since our 4d-cultures, although predominantly containing multinucleated osteoclasts, still contain some mononucleated precursor cells. However, we did not observe any decrease of RAMP1 mRNA in the M-CSF/RANKL stimulated BMM from d2 to d4. Another reason for the discrepancies might be differences in the sensitivities for the RT-PCRs.

Activation of CTR and CRLR is linked to adenylate cyclase and accumulation of intracellular cAMP. In order to assess if the receptors expressed in BMM were functional, we measured the intracellular levels of cAMP after addition of the different peptides of the CT family. We found that in M-CSF stimulated BMM, CGRP, ADM and IMD all caused an increased in cAMP, indicating that BMM express functional receptors for these peptides. These findings are in line with our results that BMM express CRLR and at least RAMP1 and RAMP2, and thereby can form the complexes constituting the receptors for these peptides. CT and CRSP did not affect the cAMP levels. This was also expected since BMM do not express the CTR. However, in these cells, AMY was able to stimulate the formation of cAMP and therefore it seems as if AMY can activate CRLR in combination with one of the RAMP proteins. This is not in line with the general view that the receptor for AMY consists of the CTR in complex with one of the three RAMP proteins. However, Dacquin et al. (2004), have reported that whereas heterozygotic CTR deficient mice exhibit a high bone mass phenotype due to an increased bone formation and an unaffected bone resorption, mice deficient in AMY exhibited the opposite, namely decreased bone mass due to increased bone resorption, without any effect on bone formation rate. If AMY only signals via the CTR, the mice deficient in CTR (Dacquin et al., 2004) would also have an affected bone resorption, as seen in the AMY deficient mice.

Therefore, it is possible that the inhibitory effect of AMY on bone resorption is mediated by a receptor other than CTR. The finding that increased bone resorption in AMY deficient mice could also be observed when these mice were breeded with CTR heterozygotic mice, further demonstrates that AMY can inhibit bone resorption independent of the CTR. When stimulating BMM with M-CSF in combination with RANKL to induce osteoclastogenesis, we found that CGRP, AMY and IMD still caused an increase in cAMP levels, showing that the receptors for these peptides remained during osteoclast development. The signalling caused by CGRP and AMY was somewhat elevated in comparison to that seen in cultures stimulated with M-CSF alone. This might be due to the appearance of CTR; according to the literature, the CTR in combination with RAMP1 has been shown to transduce some responses to CGRP as well as to AMY (review by Hay et al., 2006; Sexton et al., 2006). The presence of two receptors, capable of relaying the signal by these peptides may explain the higher response in BMM cells stimulated with both M-CSF and RANKL. In these cultures, CT and CRSP also increased the cAMP levels, demonstrating that these cells express functional CTR-based receptors, as well. Surprisingly, in BMM cultures stimulated with M-CSF and RANKL, the stimulation of cAMP caused by ADM was significantly decreased as compared to the response in cultures without RANKL. It seems as if the precursor cells, differentiating along the osteoclastic lineage, somehow lose their ability to respond to ADM (Further discussed below).

CT is known to cause a downregulation of the CTR (Samura et al., 2000), but it is, however, not known if the other peptides in the CT family are able to regulate CTR, nor is it known if the peptides can regulate the expressions of CRLR and RAMP1-3. We, therefore, examined the effects of CT, CGRP, AMY, ADM, IMD, and CRSP, on the mRNA expression of the different receptor components. We found that none of the peptides had any effect on the mRNA levels of either CRLR or the RAMPs. Surprisingly, not only peptides acting via the CTR (CT, CRSP, AMY), but also those acting via CRLR/RAMPs (CGRP, ADM, IMD and maybe also AMY in these cells) abolished RANKL induced CTR mRNA. It is interesting to note that also ADM, which does not affect osteoclast differentiation or activity, affects RANKL induced enhancement of CTR. These observations suggest the existence of not only homologous but also heterologous downregulation of the CTR.

Effects of the peptides of the CT family on osteoclastogenesis

Having established that BMM, stimulated with M-CSF and RANKL, express functional receptors for all peptides of the CT family, we examined the effects of these peptides on osteoclast differentiation. In these studies we initially used cells isolated from spleen, without any purifying step. Spleen cells are very easy to

isolate and stimulation of these cultures by M-CSF increase the proliferation and survival of the osteoclast progenitor cells. Addition of RANKL induces the differentiation of these progenitors into osteoclasts and result in the formation of TRAP positive, multinucleated cells. Addition of CT to spleen cells stimulated by M-CSF and RANKL caused an inhibition of formation of multinucleated osteoclasts and resulted in the appearance of TRAP positive mononucleated osteoclast precursor cells. The effect by CT did not induce apoptosis, nor did it affect the total TRAP activity. The effect was concentration-dependent with half maximal inhibition obtained at 10^{-12} M, which is equivalent to concentrations found in blood, indicating that these effects of CT probably occur *in vivo* as well. Our results are comparable to a study reporting that CT inhibits the formation of osteoclasts in D3 stimulated bone marrow cultures (Cornish et al., 2001). Similar to their findings we found that it was sufficient to add CT at the end of the culture period, to have the inhibitory effect, indicating that CT influence osteoclastogenesis at late stages.

Having observed this pronounced phenotype, caused by CT, we sought to find the mechanism by which CT affected M-CSF/RANKL signalling. One possibility is that the number of receptors for M-CSF and RANKL decreased. We, therefore, examined the expression of the receptors for these two effectors, c-Fms and RANK. Using conventional semi-quantitative RT-PCR, we showed that M-CSF and RANKL caused an upregulation of both c-Fms and RANK mRNA after 4 d. Whether this is an indication of an increase of the relative proportion of the osteoclast progenitor pool, or an increase in receptor density on individual cells cannot be concluded form these studies, but presumably it is a result of both events. Since RANKL induced the expression of the CTR and we have detected CTR on both mono- and multinucleated cells, we analysed the effect of CT at different time points (1-4 d). However, CT did not affect the expression of either c-Fms or RANK at any time point. These results are reasonable since CT can be added for only the last day of culture and still exert its effects.

The activation of RANK sets off several intracellular signalling pathways. These include the activation of MAP kinases, including p38, ERK1/2 and JNKs. The activation of these kinases activates several transcription factors such as NFκB and AP-1. RANK activation also leads to the activation and amplification of NFAT2, the most important transcription factor involved in osteoclast differentiation. One possible effect of CT might be on the downstream signalling, following receptor activation and, therefore, we examined the mRNA expression of the transcription factors NFAT2, the inhibitor of NFκB (IκB), as well as of two of the proteins forming the dimeric transcription factor AP-1: c-Jun and c-Fos. Expressions of all of these genes were upregulated by M-CSF and RANKL, but CT seems to be without effect at this regulatory point. The unaltered level of NFAT2 indicates that this signalling pathway has not been affected by CT since

NFAT2 activation leads to an autoamplification of NFAT2 mRNA expression. The unaltered level of IκB suggests that the NFκB signalling has not been affeted. Since NFκB binds to the promoter of IκB, an effect caused by CT on NFκB activation would subsequently have led to an altered level of IκB mRNA. Therefore, we concluded that the effect of CT does not involve the regulation of the transcription factors NFAT2 , NFκB or AP-1.

The third group of genes we decided to examine was those that are known to be important for osteoclast activity and function. We analyzed the mRNA expression of CTR, cathepsin K, TRAP, integrins α_v and β_3, MMP9, ATP6i and ClC7. All of the gene expressions, except for ClC7, were upregulated during osteoclastogenesis, but the only expression affected by the addition of CT was that of CTR, which was downregulated. These results indicate that the effects of CT occur very late during osteoclast differentiation. This is also supported by the fact that even when CT was added only for the last day of a 4-day culture period, it still inhibited the formation of multinucleated osteoclasts.

During recent years, the interactions between bone and the immune system have become more and more evident. These two systems share regulators, receptors, as well as intracellular signalling pathways. Activation of Ig-like receptors, mostly associated with B cells, T cells and NK cells, has recently been shown to be necessary for osteoclast development (Kaifu et al., 2003). The Ig-like receptors identified in the osteoclastic lineage include PIR-A, OSCAR, TREM2, SIRP-1β and NKG2D (Koga et al., 2004). These Ig-like receptors are dependent on adaptor proteins containing an ITAM motif to relay their signals. The importance of these receptor complexes for osteoclast formation has been elegantly demonstrated in knock-out mice lacking the ITAM-bearing adaptor proteins DAP12 and FcRγ. This double knock-out exhibited severe osteopetrosis due to a complete absence of osteoclasts (Koga et al., 2004; Mócsai et al., 2004). Culture of BMM derived from these double knock-outs, resulted in a large number of TRAP positive mononucleated cells. However, the ability to form multinucleated osteoclasts was disrupted. Recently, Ochi et al. (2007), have shown that signalling via FcRγ-associated Ig-like receptor PIR-A is important in TNF-α induced bone loss due to autoimmune arthritis.

The formation of mononucleated TRAP positive cells and absence of multinucleated osteoclasts, in BMM cultures derived from the $FcR\gamma^{-/-}$ $DAP12^{-/-}$ double knock-out, was very similar to the phenotype we observed after CT treatment. Therefore, we analyzed the expressions of DAP12, FcRγ and their associated receptors in spleen cells. We found that mRNA of FcRγ, DAP12 and the associated receptors OSCAR, PIR-A, TREM-2, TREM-3, NKG2D and SIRP1β were all expressed in spleen cells. Whereas PIR-A, TREM-2, TREM-3 and SIRP1β expressions were unregulated by M-CSF and RANKL, we could confirm that the expression of OSCAR was upregulated (Kim et al., 2002; So et

al., 2003) both at early and late stages of osteoclastogenesis. We could also confirm that NKG2D mRNA is upregulated (Humphrey et al., 2004) by M-CSF and RANKL. However, in contrast to our results in spleen cells, Humphrey et al. (2004) reported that RANKL increased the expression of TREM2 and TREM3 in BMM. CT did not affect the mRNA expressions of FcRγ, DAP12 or the associated receptors OSCAR, PIR-A, TREM-2, TREM-3, NKG2D or SIRP1β, neither at early stages of osteoclast formation nor at later stages in M-CSF/RANKL stimulated spleen cell cultures. The fact that we did not see any effect of CT makes sence since CT did not affect NFAT2 mRNA. Since the activation of these receptor complexes leads to the activation of calcineurin and subsequent dephosphorylation and activation of NFAT2, and since NFAT2 binds to its own promoter, thereby causing an autoamplification of mRNA expression; the mRNA expression ought to have been altered if these signalling pathways by Ig-like receptor complexes were affected by the actions of CT. However, had NFAT2 expression been affected, in addition to being a result of altered Ig-like receptor expression, the effect could have been a result of hindered signalling following receptor activation.

It is not possible to determine, for sure, if CT affects the osteoclast precursors directly, or if the effect is mediated by some other hematopoietic cells present in the spleen cell cultures. Therefore, we also used the highly enriched BMM cultures. Also in these cultures, CT caused a profound inhibition of the formation of multinucleated TRAP positive osteoclasts, without affecting the expression of FcRγ, DAP12 and OSCAR. Neither was the expression of cathepsin K, TRAP, ATP6i, integrin β_3 or MMP9 affected. Dendritic cell-specific transmembrane protein (DC-STAMP) has also been found to be highly upregulated during osteoclastogenesis, and shown to be important for osteoclast formation (Kukita et al., 2004; Yagi et al., 2005). Mice, deficient in DC-STAMP exhibit a phenotype similar to the one we have observed, with the formation of mononucleated TRAP positive cells, but a complete absence of multinucleated cells (Yagi et al., 2005). We therefore also analyzed the expression levels of DC-STAMP mRNA. We found that it was upregulated in BMM cultured in the presence of M-CSF and RANKL. However, CT did not affect the mRNA levels of DC-STAMP. The only effect of CT in the BMM was the downregulation of the CTR mRNA.

In addition to CT, the calcitonin gene family of peptides consists of five other members. AMY and CGRP have been shown to stimulate osteoblast differentiation (Cornish et al., 1995; Cornish et al., 1997; Cornish et al., 1998) and to inhibit the formation of multinucleated osteoclasts in D3 stimulated bone marrow cultures (Cornish et al., 2001). AMY and CGRP also inhibits osteoclast activity and bone resorption in organ cultures (Cornish et al., 1994), wheras ADM has no such effect (Cornish et al., 1997). Much less is known about the effects of the recently discovered CRSP and IMD. We show that CRSP and IMD, similar to CGRP and AMY, caused a pronounced decrease in the formation of

multinucleated osteoclast, resulting in many TRAP positive mononucleated cells, due to direct effects on the osteoclast precursor cells. Recently, Notoya et al. (2007) have shown that CRSP can inhibit the formation of osteoclasts in D3 stimulated co-cultures of spleen cells and stromal cells, as well as in M-CSF and RANKL stimulated bone marrow cells, but to our knowledge, this is the first report on the effects of IMD on osteoclast differentiation. In ADM treated cultures, on the other hand, no significant effects on osteoclast formation were seen. None of the peptides affected the mRNA expression of TRAP, MMP9, ATP6i or OSCAR. IMD and CRSP, the most recent members of the CT family of peptides, caused a concentration-dependent inhibition of osteoclastogenesis. These peptides did not affect cell proliferation in BMM cultures, as assessed by the MTT assay. Also with IMD and CRSP, it was sufficient to add the peptides during the last day of culture to obtain this inhibition, indicating that the effects occur late during osteoclastogenesis.

cAMP functions as a second messenger activating at least two pathways, the canonical protein kinase A signalling (PKA) and the non-canonical pathway involving the exchange protein directly activated by cAMP (Epac) signalling (Kawasaki et al. 1998; Rooij et al., 1998). CT has been linked to activation of cAMP/PKA as well as to protein kinase C/$Ca2^{+}$ signalling (Purdue et al., 2002). CT has also been shown to stimulate the phosphorylation of ERK1/2 in CTR expressing cells (Chen et al., 1998; Zhang et a., 2002), partially caused by the activation of PKC and subsequent elevation in intracellular Ca^{2+} levels. In 2002, Laroche-Joubert et al. (2002), showed that Epac is important for ERK1/2 activation. These results indicate that in addition to activating the canonical cAMP pathway, CT may also activate the non-canonical pathway involving Epac. Epac promotes exchange of GDP for GTP at the guanyl nucleotide binding site on the small G protein Rap1 (Kawasaki et al., 1998; Rooij et al., 1998). Rap1 thereafter interacts with Raf, which in turns can activate a MEK-ERK kinase cascade. Activation of ERK ½ has been shown to be essential for inhibition of osteoclast formation caused by AMY (Dacquin et al., 2004). The study showed that AMY caused a rapid and transient phosphorylation of ERK 1/2 and that in osteoclast cultures expressing a dominant form of ERK ½, AMY could not affect osteoclast formation.

We examined the effect of two different cAMP analogues: 6-MB-cAMP, which activates PKA but not Epac, and 8-pMeOPT-2'-o-cAMP, which activates Epac but does not affect PKA, on osteoclast formation in BMM cultures. Both analogues caused an inhibition of the number of TRAP positive multinucleated osteoclasts with very many TRAP positive mononucleated cells, similar to the findings in cultures treated with CT, CGRP, AMY, CRSP and IMD. These results indicate that the effects seen by CT, CGRP, AMY, CRSP and IMD may be dependent on both the canonical cAMP/PKA pathway as well as the cAMP/Epac pathway.

Effects of the peptides of the CT family on osteoclast activity

The degree of bone resorption in skeletal remodelling is not only dependent on the formation of osteoclasts, but also on the activity of mature osteoclasts. The physiological role of the peptides of the CT family in bone metabolism is not fully understood. CT is known to inhibit mature osteoclasts and bone resorption in response to high serum Ca^{2+}, but studies of genetically engineered mice have shown that mice deficient in the gene encoding CT/α–CGRP do not exhibit the expected decreased in bone mass caused by increased bone resorption. Instead, these mice have an increased bone mass due to enhanced bone formation (Hoff et al., 2002). These results are supported by the observation that mice, heterozygous deficient in CTR mice also exhibit increased bone mass (Dacquin et al., 2004). The increased bone mass observed in CT/α–CGRP deficient mice is most likely due to the absence of CT, as mice selectively lacking α–CGRP exhibit osteopenia caused by decreased bone formation (Schinke et al., 2004). Inactivation of the amylin gene, on the other hand, leads to the more expected phenotype of decreased bone mass due to increased bone resorption (Dacquin et al., 2004).

CT, CGRP and AMY have all been shown to inhibit osteoclast activity (reviewed by Zaidi et al., 2002; Lerner, 2006), and Notoya et al. (2007) have reported that CRSP treatment destroyed the actin ring in osteoclasts by a PKA-dependent mechanism. To our knowledge, hovever, there are no reports on the effects of IMD on osteoclast activity, nor are there any reports on the effects by CRSP and IMD on bone resorption. We, therefore, examined the effects of CRSP and IMD on osteoclast activity, and as a comparison we included CT, CRSP and AMY in the analyses. For these studies we used calvarial organ cultures, microdissected from neonatal mice. We first analyzed whether these organ cultures express functional receptors for the peptides. Incubation of neonatal mouse calvarial bones with CT, CGRP, AMY and CRSP resulted in enhancement of cAMP formation. There were some indications of that ADM and IMD also had an effect, however these were not significant. The results indicate that calvarial bone express receptors for at least CT, CGRP, AMY and CRSP. However, since the calvarial periosteum contains several different cell types of mesenchymal and hematopoetic origin, it is not possible to determine which cells that caused this elevation in cAMP.

After having observed the receptor expressions, we next examined the effects by the different peptides on osteoclast activity. In order to examine the effects of the peptides of the CT family on bone resorption, as a measurement of osteoclast activity, we used an *in vitro* model, measuring the release of ^{45}Ca from prelabelled neonatal calvarial bones. The bones were prestimulated with PTH or

D3 to increase the number of actively bone resorbing osteoclast prior to addition of the different peptides. We initially used a wide range of concentrations of peptides and could observe that CT, CGRP and AMY, concentration-dependently, inhibited PTH stimulated ^{45}Ca release. These results are in line with previous reports (Pietschmann et al., 1993; Cornish et al., 1994). We could also, for the first time, observe that this effect was also seen after treatment with CRSP or IMD. Salmon CT, which is known to be very potent, and therefore have been used in treatment of osteoporosis, had the highest potency of the different peptides, followed by α-CGRP, β-CGRP and AMY. IMD and CRSP had the lowest potency of the peptides but still caused the same degree of inhibition as the others. ADM did not have any significant effect on PTH stimulated ^{45}Ca release, in line with a previos report by Cornish et al. (1997). Already 3 h after addition of peptides, significant inhibition of ^{45}Ca release from the PTH stimulated bones was detected. This inhibitory effect progressively increased for 24 h, at which time point the inhibition evened out and bone gradually started to release increasing amounts of ^{45}Ca again. The fact that the inhibitory effect was so rapid indicates that it was a result of reduced osteoclast activity, and not an effect on formation of osteoclasts. CT, CGRP, AMY, CRSP and IMD, but not ADM, inhibited ^{45}Ca release also in calvarial bones prestimulated by D3. The mechanisms by which PTH and D3 exert their effects are very different. Whereas PTH binds to a membrane bound GPCR and induces an intracellular signalling cascade, D3, in complex with its receptor, functions as a ligand-activated transcription factor. The fact that the osteoclastic activity induced by both these hormones can be inhibited further indicates that the effects by CT are not interfering with the PTH and D3 simulated osteoclasts formation, but is a direct effect on the actively resorbing cells. In addition, all the peptides that inhibited hormone stimulated ^{45}Ca release (CT, CGRP, AMY, IMD and CRSP-1) also significantly decreased PTH stimulated bone matrix degradation, as assessed by the release of collagen type 1 degradation product to the culture medium.

The rapid inhibitory effect of mineral release from PTH stimulated calvaria, caused by IMD and CRSP was transient, as previously reported for CT, CGRP and AMY (reviewed by Zaidi et al., 2002; Lerner 2006). This phenomenon has been described as “escape from CT induced inhibition of bone resorption” (Wener et al., 1972). One explanation to escape has been suggested to be that the downregulation of CTR, induced by the hormone itself (Samura et al., 2000), render the osteoclasts unable to respond to further stimuli. However, since not only peptides acting via the CTR (CT, CRSP, AMY) but also peptides acting via CRLR/RAMPs (CGRP, IMD) cause this transient effect it is not likely that decreased CTR expression is the mechanism. The escape phenomenon is also seen when osteoclasts are treated with cAMP analogues as well as with the stimulators of adenylate cyclase forskolin and choleratoxin (Lerner et al., 1984; Ransjö & Lerner, 1987) These observations further indicate that the mechanism

is not related to downregulation of receptors but rather to a post-receptor mechanism.

Addition of forskolin, which increases cAMP formation through receptor independent activation of adenylate cyclase, abolished PTH stimulated ^{45}Ca release. cAMP can bind to and activate PKA as well as the Epac pathway (see above). To examine if these signalling pathways are involved in the inhibitory effect on mineral release, caused by CT, CGRP, AMY, IMD and CRSP, we added cAMP analogues with affinity to either PKA (6-monobutyryl-cAMP) or Epac (8-MeOPT-cAMP) to PTH prestimulated bones. None of these cAMP analogues could inhibit bone resorption in PTH stimulated calvarial bones, however, a cAMP analogue stimulating both these pathways, caused a decrease in bone resorption. These results indicate that cAMP signalling is involved in the effects caused by CT, CGRP, AMY, and CRSP. In addition it indicates that the both the PKA and Epac signalling pathways are needed to inhibit of bone resorption in the calvaria.

Effects of adrenomedullin on osteoclast formation and activity

The data presented in the present thesis show that ADM, in contrast to the other members of the CT family of peptides, does not affect osteoclast formation, nor bone resorption. In the calvarial bone, there were some indications of that ADM may cause an elevation in cAMP, indicating the possible presence of functional receptors in some of the cells. However, in calvarial bone cultures, it is not possible to know which cells that responds to ADM. We, therefore, also examined the capacity of ADM to enhance cAMP in BMM cultures, highly enriched in osteoclast progenitor cells. In M-CSF stimulated BMM cultures, ADM, as well as CGRP, AMY and IMD, enhanced cAMP formation. This shows that the osteoclast progenitor cells express ADM receptors. Addition of RANKL to the cultures, to induce osteoclast differentiation, decreased the responsiveness of the cells to ADM, whereas the capacity of the cells to respond to CT and CRSP was induced. These results show that during osteoclast differentiation, the cells lose their ability to respond to ADM. Interestingly, ADM has been shown to be produced in a macrophage-monocyte cell line (Kubo et al., 1998) and this has led to speculations about whether osteoclasts produce ADM as well. This could possibly be an explanation to why the osteoclasts lose their ability to respond to ADM.

According to the literature, the receptor for ADM consists of CRLR in complex with either RAMP2 or RAMP3. We have demonstrated that BMM express mRNA for CRLR as well as all three RAMP proteins, even though RAMP2 and RAMP3 are expressed at low levels. We have also detected protein expression of these receptor components. These cells could respond to ADM, similar to CGRP.

Since CGRP-stimulated cAMP accumulation was not affected during RANKL-induced osteoclastogenesis, it is not likely that the reduced responsiveness to ADM is a result of downregulation of the CRLR. In fact, mRNA analyses revealed a transient upregulation of CRLR mRNA during the initial stages of osteoclastogenesis, even though we were unable to demonstrate any differences at protein levels. At a protein level, we could not observe any differences in RAMP expression during osteoclast differentiation. Together, these findings indicate that the reason for the decreased ADM-induced cAMP response, after RANKL stimulation, is not due to decreased receptor expression. Although the mechanism remains unknown, our data strongly indicate that the absence of effect by ADM on osteoclast formation and activity is due to a decrease in ADM signalling.

Receptor expression and effects of the CT family of peptides in osteoblasts

Mice lacking CT/α–CGRP exhibit an increased bone mass due to enhanced bone formation (Hoff et al., 2002) whereas mice selectively lacking α–CGRP exhibit osteopenia caused by decreased bone formation (Schinke et al., 2004). In addition, both AMY and ADM stimulates osteoblast proliferation (Cornish et al., 1995; Cornish et al., 1997; Cornish et al., 1998). We, therefore, sought to examine the expression of the components forming the receptors for these peptides in a primary osteoblast-like cell line.

For these studies, we used primary calvarial osteoblasts, isolated from 2-to-3-day-old mice (see methods). These cells are CT non-responsive and exhibit an osteoblastic phenotype as assessed by their cAMP-responsiveness to parathyroid hormone (PTH) and the capacity to form mineralized bone noduli (data not shown). The cells spontaneously differentiate into bone forming osteoblasts over time. To further enhance this process, we stimulated the cultures with BMP-2, in some experiments.

Analyses of mRNA showed that CRLR was slightly enhanced during spontaneous differentiation of these cells and also that all three RAMPs were expressed. This suggests that cells differentiating along the osteoblastic lineage become more sensitive to stimulation or inhibition of the peptides signalling via the CRLR-based receptor complexes. Our results are in line with previous reports that osteoblast-like cells express CRLR and the RAMP proteins (Uzan et al., 2004). The expression of osterix and ALP was clearly enhanced by the addition of BMP-2 to the cultures. Most surprisingly, we also observed that the CTR was spontaneousoly upregulated after a period of time, and that this expression was profoundly enhanced by the addition of BMP2.

To examine whether these receptors were functional, we measured the intracellular cAMP levels after 2 and 12 d of culture. At 2 d, CGRP, AMY, ADM and IMD all caused an increase in cAMP levels. This is in line with our findings that the osteoblastic cells express mRNA for CRLR and all three RAMP proteins. CT and CRSP did not affect the intracellular levels of cAMP. This was expected since, at this time point, no CTR mRNA was detected. At d12, all peptides stimulated cAMP production. At this time point, we had detected CTR mRNA and therefore, these results make sense. However, the induction of cAMP caused by CGRP, AMY, ADM and IMD were much weaker at 12 d as compared to 2 d. This is hard to explain since the mRNA analyses did not reveal any downregulation of CRLR or RAMP2 or RAMP3. RAMP1 was transiently downregulated and, during the time period analyzed, the expression did not reach its original levels. However, according to the mRNA analyses, CGRP, AMY, ADM and IMD should still induce the same response after 12 d. The explanation to this discrepency in our results requires additional analyses, presumably at a protein level.

CTR expression has been detected in several tissues, but is mostly associated with the osteoclast precursor cells and mature osteoclasts (Nicholas et al., 1986). Osteoblasts have, however, not been shown to express this receptor and therefore, we found it necessary to examine whether our osteoblast cultures might contain some contaminating cells of the osteoclastic lineage. Analyses of mRNA revealed that at least a portion of the cells in these cultures expressed the cathepsin K and, that the addition of BMP-2 induced the expression of the osteoclastic genes MMP-9 and TRAP as well. Although cathepsin K also seems to be expressed in osteoblasts (Mandelin et al., 2005), the most likely explanation to these findings is that BMP-2 stimulates osteoclast formation. To address this, we examined the effects of BMP-2 on the expression of RANKL, since RANKL is expressed by the osteoblasts and indispensible for osteoclastogenesis to occur. We also analyzed the expression of OPG, the decoy receptor for RANKL. We found indications of that BMP-2 caused a slight increase in RANKL, detectable after 6 days and more evident after 12-18 d. In parallel, the expression of OPG seemed slightly downregulated by BMP-2 after 6 d. Even if these regulations are very small *per se*, the addition of BMP-2 may slightly augments the RANKL/OPG ratio which could explain why BMP-2 causes a stimulation of osteoclast differentiation. These differences of mRNA levels were, however, not significant (except for RANKL and OPG, d18) and further studies needs to be done to verify these results. Another explanation to the induction of CTR, TRAP and MMP-9 is that BMP-2 may have a direct stimulatory effect on osteoclast progenitor cells.

Since there seemed to be a small proportion of osteoclasts/osteoclast precursor cells, we also examined the expression of the CTR, CRLR and all three RAMP proteins in the osteoblastic cell line, MC3T3-E1. In these cultures we could still

detect the CRLR and the three RAMPs, but there was no induction, neither spontaneous nor BMP-2 induced, of the CTR mRNA. This indicates that the expression of CTR, TRAP and MMP-9 seen in the osteoblastic cell cultures probably do originate from osteoclast progenitor cells.

These results show that althogh the osteoblastic-like cells, obtained by using the modified time sequential enzyme-digestion technique described by Boonekamp et al. (1984), are relatively pure, there is a risk that also other cells are present in the cultures derivded from calvarial periosteum. In light of these results, in studies of effects on mRNA expression in osteoblasts, the best alternative, for the time being, is to use a cell line, such as MC3T3-E1.

CONCLUSIONS

Data presented in this thesis show that osteoclast precursor cells express mRNA for the CRLR and all three RAMP proteins, and that CTR mRNA is induced during osteoclastogenesis. These mRNA species are also transcribed into protein, and form functional receptors for all the members of the CT family of peptides.

CT, CGRP, AMY, CRSP and IMD inhibit the formation of TRAP positive multinucleated osteoclasts without affecting a number of genes known to be important for differentiation or function. In the absence of TRAP positive multinucleated cells, we observed the presence of many mononuceated TRAP positive cells. This indicates that the inhibitory effects observed, presumably occurs late in differentiation, probably at the fusion step. Also, it was sufficient to add the peptides (CT, CRSP and IMD) for the the last day of a 4-day culturing period to obtain the same degree of inhibition as when the peptides were present during the whole time period, further supporting that the effect occurs late in differentiation.

CRSP and IMD, in resemblance to CT, CGRP and AMY, inhibit bone resorption, both in calvarial organ cultures and in BMM cultures.

The lack of effect of ADM on osteoclast differentiation and activity is most likely due to a decreased ADM signalling as osteoclastogenesis occur. However, there where no changes in CRLR nor RAMP1-3 protein levels and, the mechanism remains unknown.

Finally, we have observed the mRNA expression of CRLR and all three RAMP proteins in an osteoblastic cell line.

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