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DEVELOPMENTAL BIOLOGY

Developmental Biology 261 (2003) 537–547

www.elsevier.com/locate/ydbio

Deletion of the gene encoding c-Cbl alters the ability of osteoclasts to migrate, delaying resorption and ossification of cartilage during the development of long bones

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Received for publication 15 January 2003, revised 23 April 2003, accepted 6 May 2003

Abstract

During development of the skeleton, osteoclast (OC) recruitment and migration are required for the vascular invasion of the cartilaginous anlage and the ossification of long bones. c-Cbl lies downstream of the vitronectin receptor and forms a complex with c-Src and Pyk2 in a signaling pathway that is required for normal osteoclast motility. To determine whether the decreased motility we observed in vitro in c-Cbl$^{-/-}$ OCs translated into decreased cell migration in vivo, we analyzed the long bones of c-Cbl$^{-/-}$ mice during development. Initiation of vascularization and replacement of cartilage by bone were delayed in c-Cbl$^{-/-}$ mice, due to decreased osteoclast invasion of the hypertrophic cartilage through the bone collar. Furthermore, c-Cbl$^{-/-}$ mice show a delay in the formation of secondary centers of ossification, a thicker hypertrophic zone of the growth plate, and a prolonged presence of cartilaginous remnants in the spongiosa, confirming a decrease in resorption of the calcified cartilage. Thus, the decrease in motility of c-Cbl$^{-/-}$ osteoclasts observed in vitro results in a decreased ability of osteoclasts to invade and resorb bone and mineralized cartilage in vivo. These results confirm that c-Cbl plays an important role in osteoclast motility and resorbing activity.

Introduction

The product of the c-Cbl proto-oncogene and other members of the Cbl family have evoked much interest in recent years, not only because some altered forms of c-Cbl promote tumorigenesis but also because of findings that relate to their structure and function. All Cbl proteins share a unique domain that recognizes phosphorylated tyrosine residues that are present on activated tyrosine kinases (Galisteo et al., 1995; Lupher et al., 1996) and a RING domain that mediates the recruitment of ubiquitin-conjugating enzymes to, and the multiubiquitination of activated receptor (RTKs) and nonreceptor (NRTKs) tyrosine kinases (Joazeiro et al., 1999; Naramura et al., 1998; Ota et al., 2000; Ota and Samelson, 1997; Yokouchi et al., 1999, 2001). Thus, Cbl proteins function by specifically targeting activated RTKs and NRTKs and mediating their downregulation, thereby providing a means by which signaling processes can be negatively regulated (Joazeiro et al., 1999; Levkowitz et al., 1999; Sanjay et al., 2001a; Yokouchi et al., 1999). However, Cbl is also involved in positive signaling events through its capacity as a multidomain adaptor protein that is required for functions associated with cell spreading in response to integrin engagement, and with bone resorption (Meng and Lowell, 1998; Ojaniemi et al., 1997; Scaife and Langdon, 2000; Tanaka et al., 1996; Zell et al., 1998).

The ability of cells to migrate is an essential physiological process, whose molecular mechanisms are so far only partially understood. In highly motile cells, such as macrophages, trophoblasts, and osteoclasts, cell motility is achieved through the rapid, highly coordinated assembly

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doi:10.1016/S0012-1606(03)00299-9

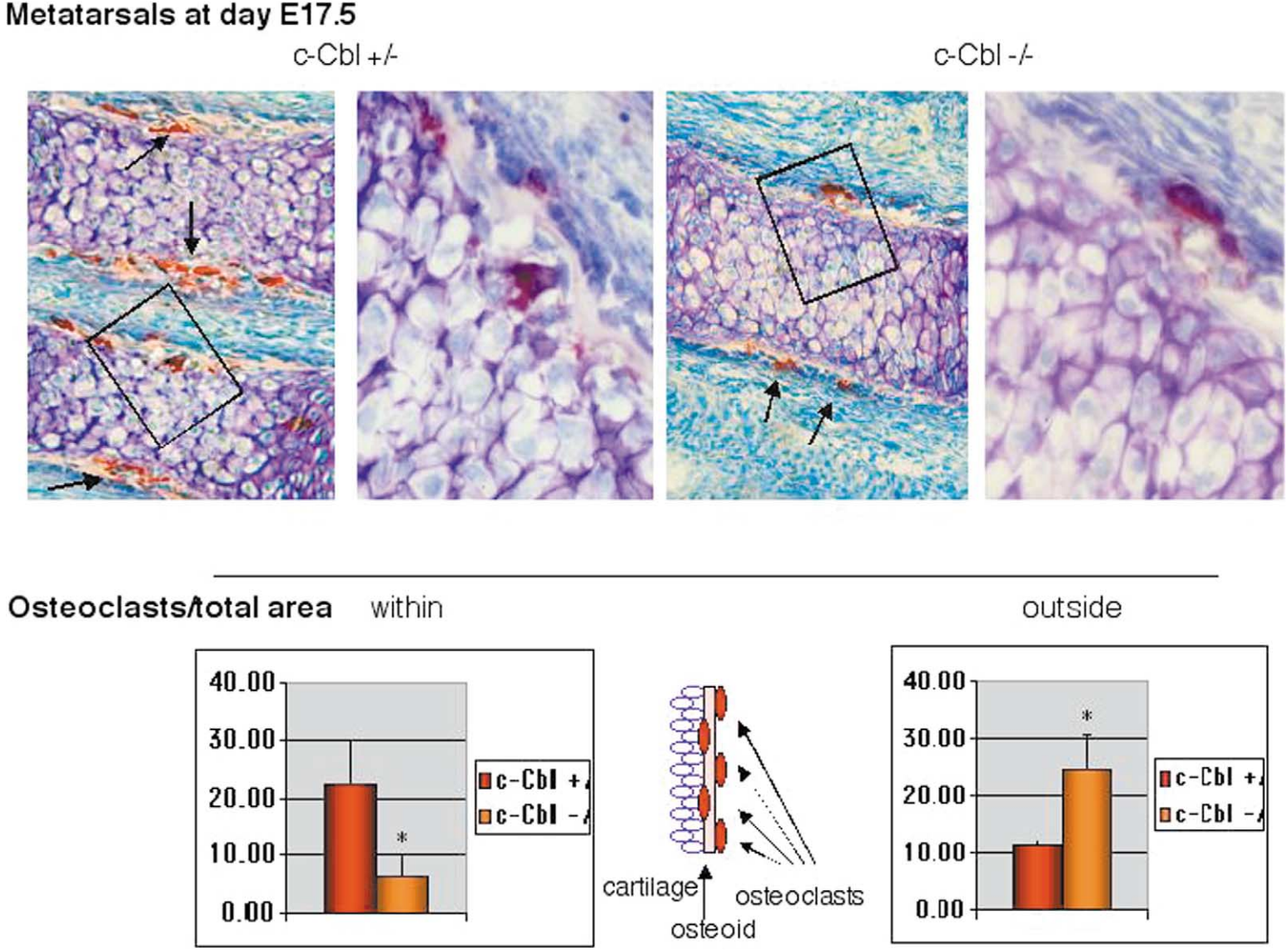

Fig. 1. Metatarsals from E17.5 c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ mouse embryos. In c-Cbl$^{+/-}$ E17.5 metatarsals, OCs have already crossed the metatarsal bone collar and begun resorbing the hypertrophic cartilage, whereas in c-Cbl$^{-/-}$ E17.5 metatarsals, OCs are found almost exclusively on the perichondral surface of metatarsals. Histomorphometric analysis of E17.5 c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ metatarsals showed that the number of osteoclasts within the bone collar was significantly reduced in c-Cbl$^{-/-}$ metatarsals at this age ($P < 0.05$), compared with their heterozygous littermates, whereas the number of osteoclasts outside the bone collar was increased ($P < 0.05$). The total number of osteoclasts was unchanged.

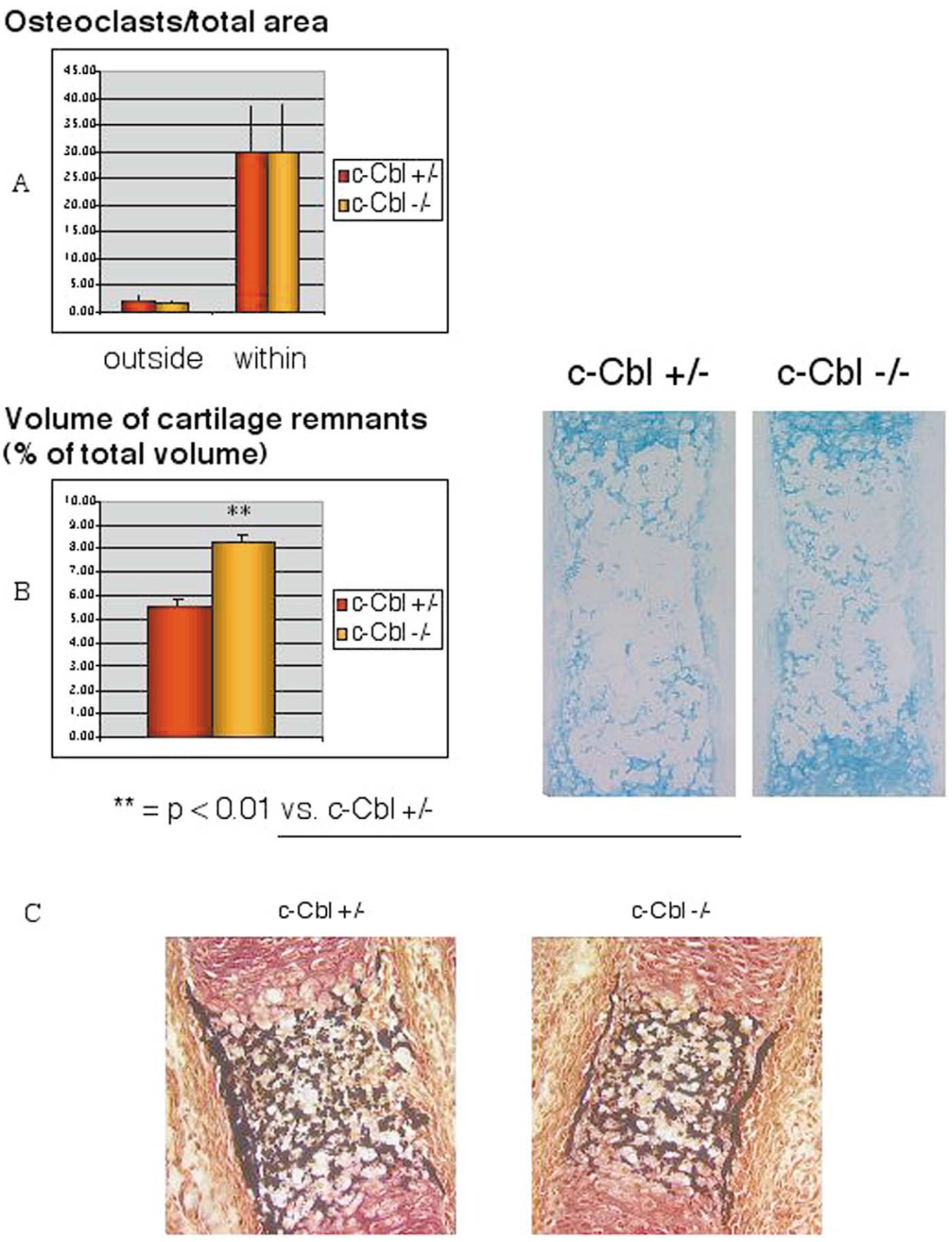

Fig. 2. (A, B) Histomorphometrical analysis and alcian blue staining of E19.5 c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ metatarsals. Both in c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ mouse embryos, almost all osteoclasts are found in the forming early marrow cavity (A; left, outer OCs; right, inner OCs); however, the volume of the cartilage remnants detected by alcian blue staining is significantly higher in c-Cbl$^{-/-}$ mice than in the heterozygous animals ($P < 0.01$) (B, C). Von Kossa staining of E17.5 c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ mouse metatarsals. The mineralization of the hypertrophic cartilage is comparable in c-Cbl$^{+/-}$ and c-Cbl$^{-/-}$ mice.

and disassembly of dynamic attachment structures called podosomes (Marchisio et al., 1984, 1987; Zambonin-Zallone et al., 1982), which occurs within minutes (Stickel and Wang, 1987).

Osteoclasts are multinucleated giant cells whose physiological role is to resorb the extracellular bone matrix (Baron et al., 1993; Teitelbaum, 2000). This role is essential for bone morphogenesis, repair, and maintenance. As osteoclasts move across bone surfaces, they must rapidly attach to and release from the extracellular matrix. The speed of podosome assembly and disassembly allows osteoclasts to generate high rates of motility. The molecular events at the basis of these fast processes are now beginning to be elucidated.

Attachment of the osteoclast to the substrate occurs mainly via the $\alpha_v\beta_3$ integrin receptor, which has been shown to be the major integrin in osteoclasts (McHugh et al., 2000; Zambonin Zallone et al., 1989). We have previously shown that the engagement of $\alpha_v\beta_3$ triggers the formation of a complex involving c-Cbl and the nonreceptor tyrosine kinases Pyk2 and c-Src, and that c-Cbl is a major regulator of the activity of this molecular complex in a signaling pathway that regulates cell adhesion and motility. OCs that lack either c-Src or c-Cbl display decreased migration in vitro (Sanjay et al., 2001b).

Osteoclast motility is required not only during the bone resorption process that occurs in remodeling adult bone but also for osteoclast (OC) recruitment and migration through the perichondrium and the bone collar that is required for the vascular invasion of the cartilaginous anlage and the formation of the primary and secondary centers of ossification during the process of endochondral bone morphogenesis. Migration is essential in order for OC precursors to reach the bone collar and for newly formed OCs to penetrate the mineralized hypertrophic cartilage during long bone development.

To determine whether the decreased motility we observed in vitro reflected decreased cell migration in vivo and to better determine the role of osteoclast migration in developmental processes of the skeleton, we analyzed these developmental events in c-Cbl knockout mice. Analysis of embryonic metatarsals showed that the initiation of vascularization and replacement of cartilage by bone are delayed in these mice as a consequence of the decreased ability of osteoclasts to invade the hypertrophic cartilage through the bone collar. Furthermore, in a Boyden chamber assay, osteoclasts isolated from c-Cbl$^{-/-}$ mice displayed a significant decrease in invasion ability compared with wild-type osteoclasts. Although a complete histomorphometric analysis failed to demonstrate any significant change in adult c-Cbl$^{-/-}$ mice, differences are evident in these mice from day 9 through day 18 of postnatal life. Specifically, there is a delay in the formation of the secondary center of ossification in the tibia proximal epiphysis, a thickening of the hypertrophic zone of the growth plate and persistant cartilaginous remnants in the spongiosa, all processes that are dependent on osteoclast invasion of the mineralized hypertrophic cartilage and constitute pathognomonic signs of decreased resorbing activity. Thus, c-Cbl plays an important part in osteoclast motility and bone resorption during skeletal development in vivo.

Methods

Generation and identification of c-Cbl deficient mice, sample preparation, and histological analysis

c-Cbl$^{-/-}$ mice were generated and identified as previously reported (Naramura et al., 1998). For histological and histomorphometrical analysis, c-Cbl$^{-/-}$ mice and wild-type and heterozygous littermates were sacrificed by cervical dislocation or halothane inhalation at days E17.5, E19.5, P9, P10, and P18 and at 12 weeks of age (the latter previously injected with calcein 30 mg/kg, 10 and 3 days before sacrifice), tissues were fixed in 4% formalin, then either frozen or embedded in methylmethacrylate as described (Sims et al., 2000). From frozen samples, 6-micron sections were either stained for TRAP using an acid phosphatase detection kit (Sigma Chemical Co., St. Louis, MO, USA) and counterstained with toluidine blue, or stained with the Von Kossa method for calcified tissues and counterstained with methyl green. From methylmethacrylate-embedded samples, 5-micron sections were stained with toluidine blue and 10-micron sections were coverslipped unstained for dynamic measurements. Histomorphometric analysis was performed with an Osteomeasure system (Osteometrics Inc., Atlanta, GA, USA), using standard procedures (Parfitt et al., 1987) to assess changes in bone structure and remodeling. Tibial sections were measured in the proximal metaphysis beginning 340 μm below the chondro-osseous junction, in a region that corresponds to the secondary spongiosa. In embryonic metatarsals, the numbers of large TRAP+ cells were determined both outside the bone collar and within, in contact with and resorbing the hypertrophic cartilage. All histomorphometric analyses were performed in a blind fashion.

Preparation of osteoclasts and osteoclast-like cells

Authentic osteoclasts were obtained from the long bones of 2- to 4-day-old neonatal mice. Bones were dissected free of adherent tissues, placed in α-MEM containing 5% FBS, and minced into small pieces. After vigorous pipetting to release osteoclasts, the bone particles were allowed to sediment for 30 s and the remaining cell suspension containing osteoclasts was seeded onto serum-coated coverslips. To obtain large numbers of cells for biochemical analyses, osteoclast-like cells (OCLs) were generated in the murine coculture system (Tanaka et al., 1996) by culturing neonatal primary calvarial osteoblasts with spleen and marrow cells in the presence of 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]

and PGE_2. Primary calvarial osteoblasts were isolated from neonatal mouse calvaria after sequential digestion with 0.1% collagenase and 0.2% dispase (Calbiochem), and then cultured in α-MEM containing 10% FBS. Cells obtained from spleens and bone marrow cells from c-$Cbl^{-/-}$ or c-$Cbl^{+/-}$ mice (2×10^8 cells) were cocultured with osteoblastic cells in α-MEM containing 10% FBS, 10^{-8} M $1,25(OH)_2D_3$, and 10^{-6} M PGE_2 (Sigma-Aldrich Co., St. Louis, MO). After 4–5 days in culture, OCLs were purified by removing the osteoblast layer by repeated pipetting of media over the surface of the cell layer. Suspensions of serum-starved OCLs (1% FCS overnight) were obtained by treating the purified OCLs with 10 mM EDTA for 5 min at 37°C. Cells were then flushed off the culture dishes, washed once in serum-free α-MEM, and resuspended in serum-free medium.

Osteoclast isolation and invasion assay

For invasion experiments with c-Cbl-positive and c-Cbl-negative osteoclasts, authentic osteoclasts were generated from the marrow of c-$Cbl^{+/?}$ and c-$Cbl^{-/-}$ mice and cocultured with primary osteoblasts in the presence of $1,25(OH)_2D_3$ (Akatsu et al., 1992). They were detached by sequential treatment with 0.1% bacterial collagenase (wt/vol) plus 0.1% dispase (wt/vol), and 0.5% trypsin (wt/vol), washed, resuspended in α-MEM, and seeded onto type I collagen-coated membranes of culture inserts (Sato et al., 1998). These culture inserts were then placed in 12-well plates containing α-MEM supplemented with 0.1% albumax. After an overnight culture, the cells were stained for TRAP. Invasion was determined by counting osteoclasts or osteoclast extensions that had reached the lower surface of the membrane after removal of the cells from the upper surface of the membranes (Sato et al., 1998).

Coimmunoprecipitation and Western blotting

Lysates were centrifuged for 30 min at 4°C at 16,000 *g*. Supernatants were used for immunoprecipitation assays. Typically, 5 μg of antibody was added to 500 μg of protein lysate and incubated at 4°C for 1 h. Protein-G agarose slurry (40 μl) was added and the incubation continued for another 1 h. The immune complexes on the beads were washed three times in mRIPA buffer and once in PBS. Beads were boiled in 2× SDS-PAGE buffer and samples were electrophoresed on 8% SDS-PAGE gels. Proteins were then transferred to nitrocellulose membranes (BA85; pore size, 0.45 μm; Schleicher & Schuell). To verify the quality of transfer, proteins were visualized on the filters by staining with 0.2% Ponceau S in 3% trichloroacetic acid. To block nonspecific binding, the filters were incubated for 2 h at room temperature in 5% milk, TBST buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Antigens were visualized by immunoblotting with the appropriate primary antibody (1:1000 dilution), and then a horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody. All blots were developed by using enhanced chemiluminescence reagents from Amersham Pharmacia Biotech. Densitometry of the bands was performed by using the Scion Image 1.62 program, and the values were normalized to the density of the actin band in the reprobed blot.

Statistical analysis

Statistical analysis was performed by using ANOVA, with *P* values less than 0.05 (*) accepted as significant; error bars represent standard deviations.

Results

It is well established that, in wild type mouse embryos at day E17.5, osteoclasts have already crossed the metatarsal bone collar and begun resorbing the hypertrophic cartilage to form the early marrow cavity, as we observed in c-$Cbl^{+/-}$ embryos (Fig. 1). However, in c-$Cbl^{-/-}$ littermate embryos at this time in development, osteoclasts were found almost exclusively on the perichondral surface of metatarsals, demonstrating that this process is delayed in these mice. This observation was confirmed quantitatively by histomorphometry. Metatarsals from c-$Cbl^{+/-}$ and c-$Cbl^{-/-}$ E17.5 mouse embryos were analyzed to determine the numbers of osteoclasts localized outside the bone collar or within, in contact with, and resorbing the hypertrophic cartilage (Fig. 1). While the total number of osteoclasts in the c-$Cbl^{-/-}$ metatarsals was not different from the number in the c-$Cbl^{+/-}$ specimens, the number of osteoclasts within the bone collar was significantly reduced in c-$Cbl^{-/-}$ metatarsals at this age, compared with their heterozygous littermates, whereas the number of osteoclasts remaining outside the bone collar was increased. The number of mononuclear osteoclast precursors, only found outside the bone collar in both groups, was unchanged (data not shown). By day 19.5, almost all osteoclasts were found in the forming early marrow cavity in both c-$Cbl^{+/-}$ and c-$Cbl^{-/-}$ mouse embryos, although the alcian blue-stained cartilage remnants were more conspicuous in c-$Cbl^{-/-}$ than in c-$Cbl^{+/-}$ metatarsals (Fig. 2A and B). This observation was confirmed quantitatively by histomorphometrical analysis, which showed that the volume of the cartilage remnants in the early marrow cavity was significantly higher in c-$Cbl^{-/-}$ mice than in heterozygotes, again indicating that the ossification of the primary center is delayed (Fig. 2B). This delay was not due to a lack of mineralization of the hypertrophic cartilage in the embryonic metatarsals, a mandatory step for osteoclast recruitment and matrix resorption, which was similar in the c-$Cbl^{+/-}$ and c-$Cbl^{-/-}$ mice, as shown by Von Kossa staining of day E17.5 metatarsals (Fig. 2C).

The formation of the secondary center of ossification in the epiphysis of long bones, a process that is also dependent on invasion of cartilage by osteoclasts, was also delayed in

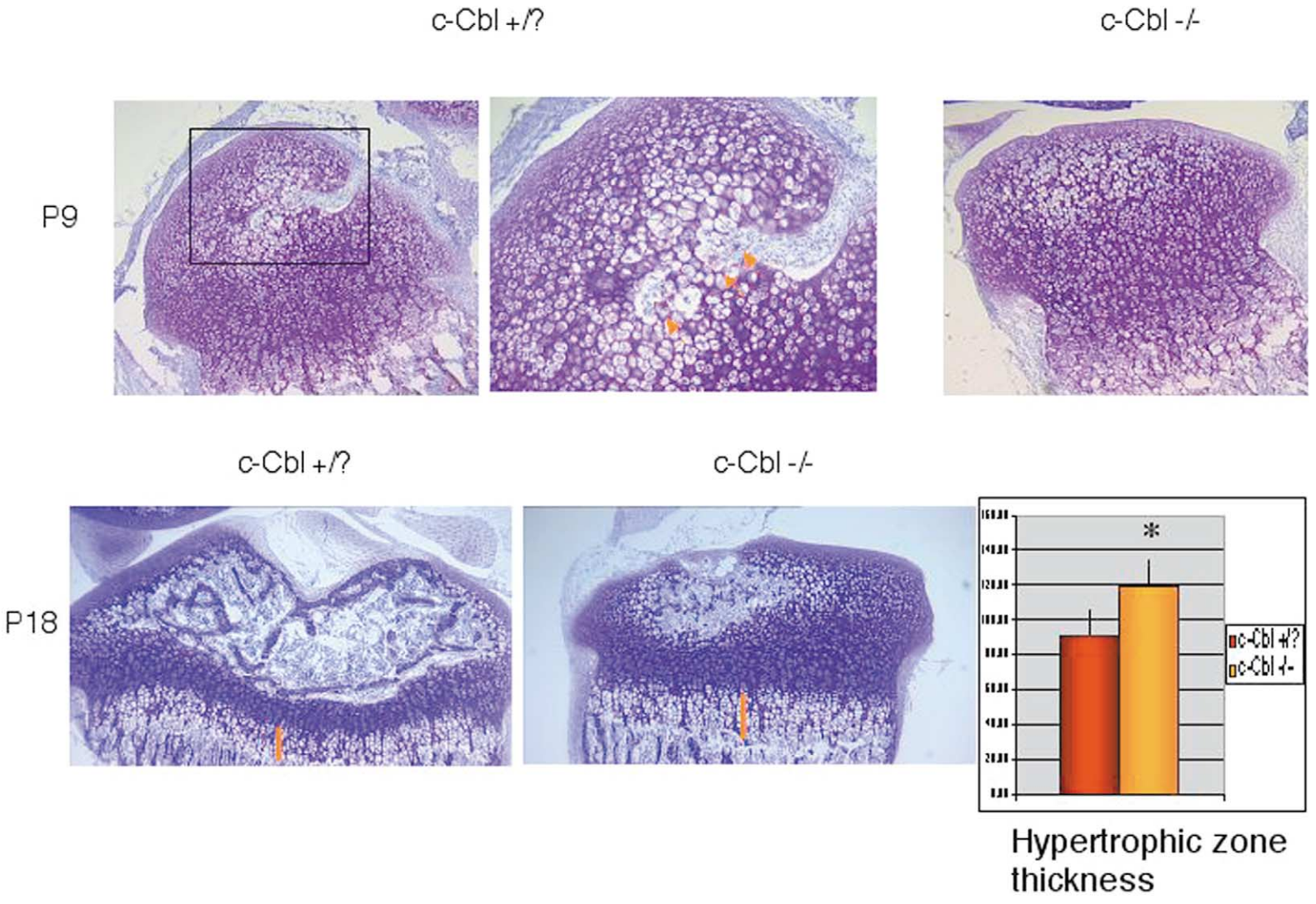

Fig. 3. Deletion of c-Cbl causes a delay in the formation of the secondary center of ossification. At day P9 (top), tibia epiphyses of c-Cbl$^{+/?}$ mice are being invaded by osteoclasts (arrows), along with endothelial cells, while only hypertrophic chondrocytes are found in the tibia epiphyses of their c-Cbl$^{-/-}$ littermates (TRAP staining and toluidine blue counterstaining). This delay is still present at day P10. At day 18 after birth, the tibia epiphysis is extensively ossified and vascularized, and a true mature marrow cavity is formed in c-Cbl$^{+/?}$ mice, whereas in their c-Cbl$^{-/-}$ littermates, this process is still at a very early stage. At this time, the hypertrophic zone (red bars) of the growth plate is thicker in c-Cbl$^{-/-}$ mice than in their c-Cbl$^{+/?}$ littermates (bottom right panel), also suggesting a slowing of resorption of the calcified cartilage.

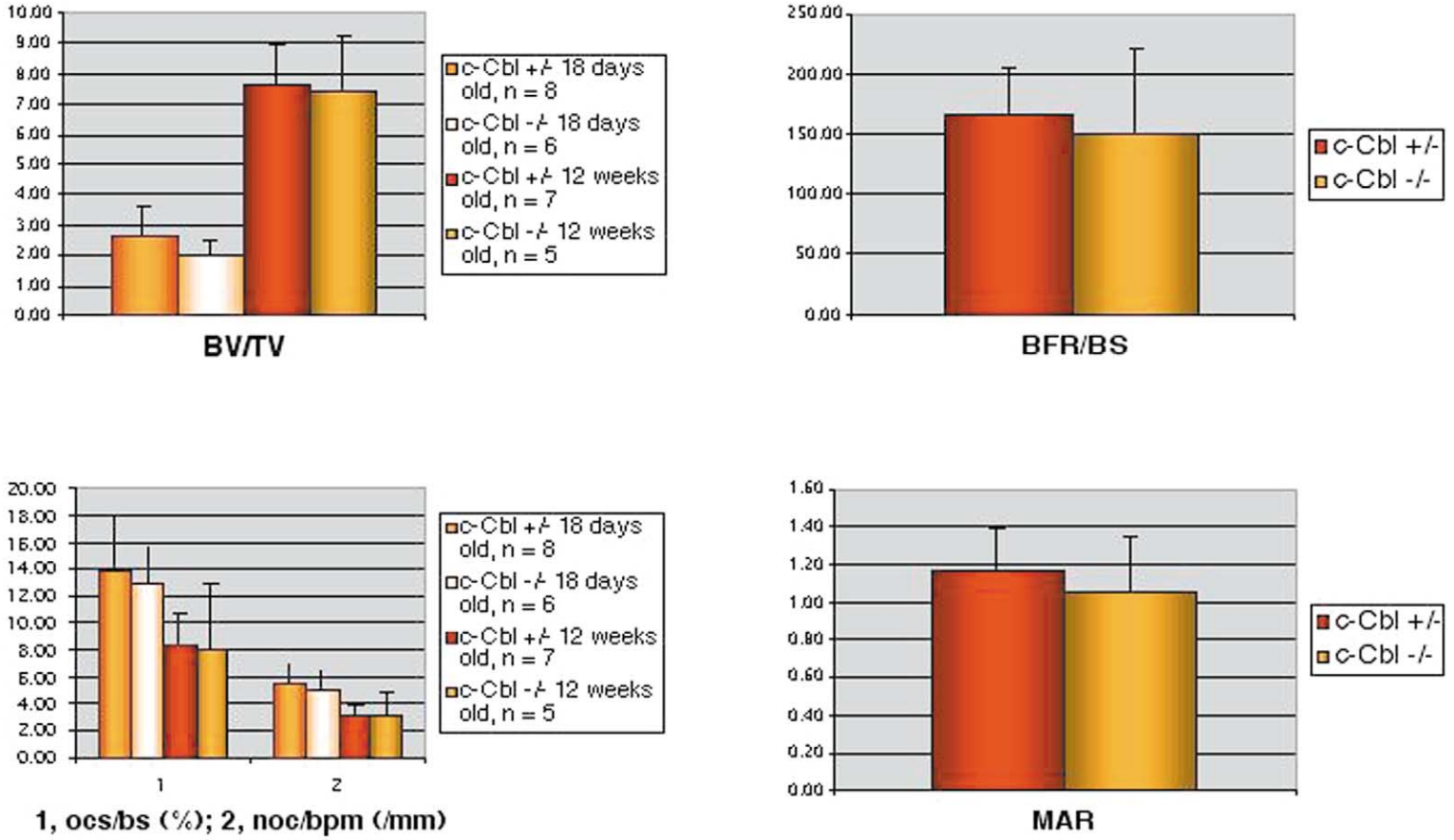

Fig. 4. Histomorphometry of c-Cbl$^{+/?}$ and c-Cbl$^{-/-}$ mice. Histomorphometrical analysis performed on tibias' secondary spongiosas failed to show any significant difference between c-Cbl$^{+/?}$ and c-Cbl$^{-/-}$ mice at either 18 days or 12 weeks of age. All standard parameters were measured; here, we report trabecular volume (BV/TV, %), osteoclast surface (OcS/BS, %) and number (OcN/BPm, [/mm]), bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/year), and mineral apposition rate (MAR, μm/day).

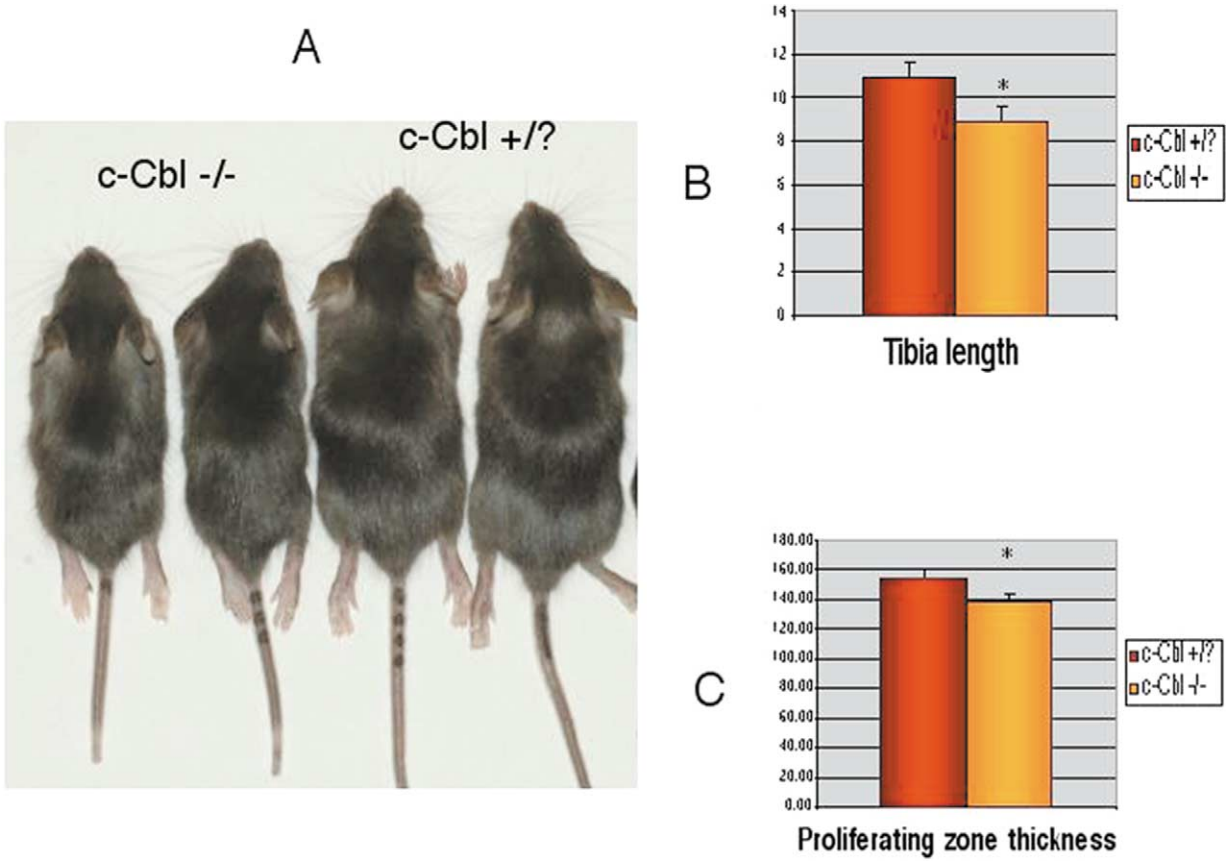

Fig. 5. Growth is transiently delayed in c-Cbl$^{-/-}$ mice. At 18 days of age, c-Cbl$^{-/-}$ mice are smaller than their wild type littermates (A), and their tibias are shorter (B). A decrease in the thickness of the proliferative zone of the growth plate at 10 days of age was also observed (C).

c-Cbl-deficient mice, as shown in Fig. 3. At day 9 after birth, in wild type and heterozygous mice, the hypertrophic cartilage in the tibia epiphysis was being invaded by osteoclasts and endothelial cells. In contrast, there was no sign of this process of invasion at this time (Fig. 3), nor 1 day later (data not shown) in the c-Cbl$^{-/-}$ littermates. At day 18 after birth, the tibia epiphysis in wild type and heterozygous mice was extensively ossified and vascularized, and a true mature marrow cavity was present, whereas in their c-Cbl$^{-/-}$ littermates, this process was still at a very early stage. At this age, the hypertrophic zone of the growth plate was also thicker in c-Cbl$^{-/-}$ mice, again suggesting a slowing of osteoclast resorption of the calcified cartilage (Fig. 3). However, in contrast with the differences observed in embryos and young mice, and consistent with published reports (Murphy et al., 1998), a complete histomorphometric analysis of bones from adult mice failed to demonstrate significant differences in any parameters-between the c-Cbl$^{-/-}$ mice and their c-Cbl$^{+/?}$ littermates (Fig. 4).

We also found that c-Cbl$^{-/-}$ mice displayed a transient delay in growth that was first detectable at day P10 (data not shown) and was still seen at day P18 (Fig. 5). Again, adult c-Cbl-null mice were indistinguishable from their wild type littermates. This delay in growth occurred simultaneously with a transient lag in the growth of long bones and thinning of the proliferating zone of the growth plate (Fig. 5). No changes were observed in the tibial metaphyseal spongiosa (Fig. 4, and data not shown).

Consistent with the retarded invasion of osteoclasts across the periosteum and the bone collar into the mineralized cartilage, freshly isolated c-Cbl-deficient osteoclasts displayed a decrease of about 50% compared with the controls ($P < 0.01$) in their ability to migrate through collagen type I-coated chemotaxis membranes (Fig. 6). Since this system monitors the ability of osteoclasts to invade a collagenous tissue, it is clearly more relevant to our in vivo model than the migration on glass coverslips reported earlier (Sanjay et al., 2001b). While the assay system does not fully reproduce the mineralized matrix encountered by osteoclasts and their precursors in vivo, the defect that we observed in this assay is consistent with the phenotype we observed in vivo in the process of osteoclast migration through the periosteum and bone collar (Fig. 1), which at that stage is mainly hypomineralized Col-I matrix.

Deletion of both c-Cbl and the highly homologous family member Cbl-b results in embryonic lethality by day 10 (Liu and Gu, 2002; Naramura et al., 2002), indicating that the two proteins share critically important functions. Redundancy of function could explain, at least in part, the relatively mild phenotype of the c-Cbl$^{-/-}$ mice and their

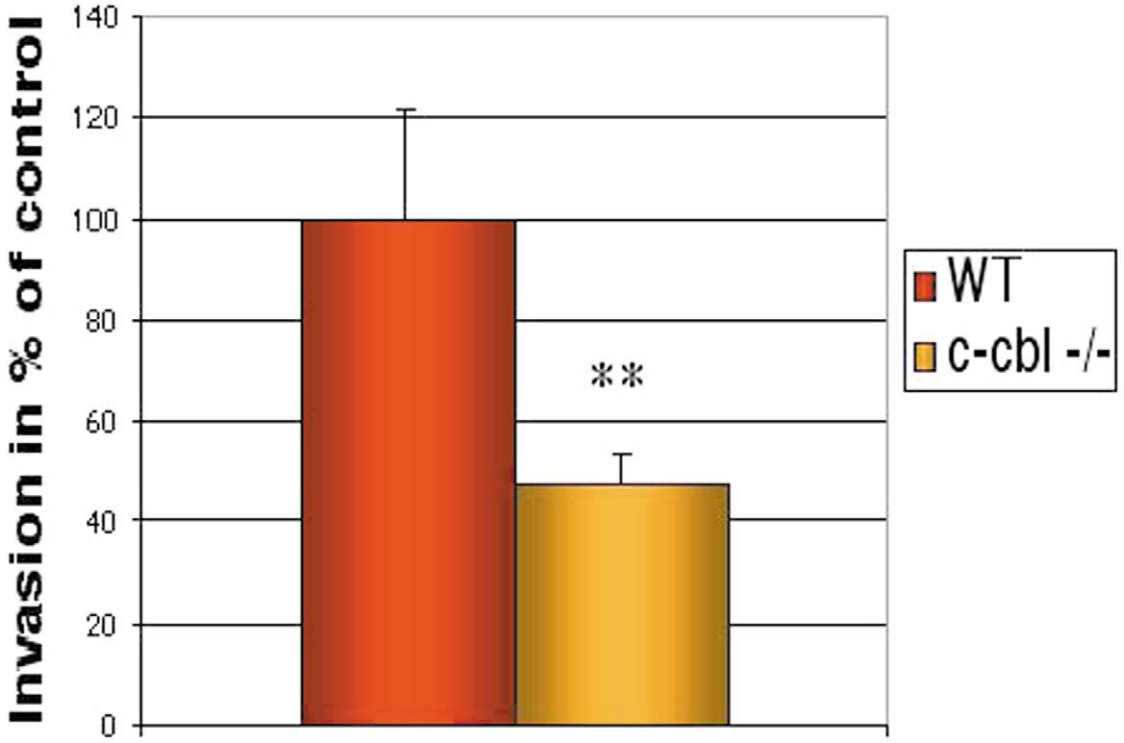

Fig. 6. Migration of c-Cbl$^{-/-}$ osteoclast-like cells through a collagen type I-coated membrane was reduced by about 50% relative to wild type ($P < 0.01$, pool of two separate experiments performed in triplicate).

recovery by adulthood, especially if the expression of Cbl-b increases as a consequence of the absence of c-Cbl. Western blot analysis performed on cell lysates from osteoclast-like cells isolated from c-Cbl$^{+/?}$ and c-Cbl$^{-/-}$ adult mice in fact showed that expression of Cbl-b is increased more than twofold in the latter (Fig. 7), suggesting that a compensation process may indeed take place with age.

Discussion

Our previous studies have provided evidence that the proto-oncoprotein c-Cbl plays an important role in osteoclast motility downstream of integrins Pyk2 and Src (Sanjay et al., 2001b; Tanaka et al., 1996), suggesting that bone resorption would be compromised in c-Cbl$^{-/-}$ mice. Others have reported, however, that there is no obvious bone phenotype in c-Cbl$^{-/-}$ mice (Murphy et al., 1998). Indeed, the more detailed analysis of the bone phenotype of adult c-Cbl$^{-/-}$ mice performed in this study showed that the deletion of c-Cbl does not affect the normal turnover and remodeling of the adult bone. Given that there are several members in the Cbl family, it is possible that another family member such as Cbl-b compensates the absence of c-Cbl, and we show here that the expression of Cbl-b is indeed increased in c-Cbl$^{-/-}$ osteoclasts. However, despite the absence of an alteration in bone remodeling parameters in c-Cbl$^{-/-}$ mice, our previous in vitro studies (Sanjay et al., 2001b) clearly demonstrated a mild but significant decrease in osteoclast motility in c-Cbl$^{-/-}$ cells, although the defect was less pronounced than the decreased osteoclast motility of c-Src$^{-/-}$ osteoclasts (Sanjay et al., 2001b), which led to a characteristic osteopetrotic phenotype (Soriano et al., 1991).

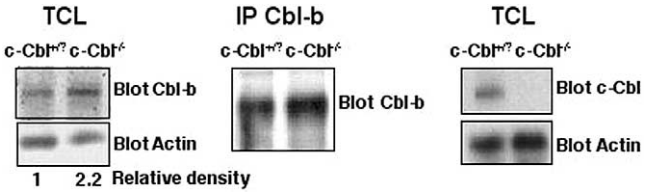

Fig. 7. Primary osteoclast-like cells (OCLs) were generated by coculturing bone marrow from 4- to 7-week-old c-Cbl$^{-/-}$ and c-Cbl$^{+/?}$ mice. Total cell lysates (TCL) were used for immunoprecipitation (i.p.) and Western blot (WB) analysis. The homologous family member, Cbl-b, was found to be overexpressed in c-Cbl$^{-/-}$ OCLs.

In the present study, we further explored the role of c-Cbl in osteoclast motility and activity by examining stages of skeletal development that are more critically dependent on the ability of these cells and their precursors to migrate. During the development of long bones, chondrocytes in the anlage proliferate and differentiate to become hypertrophic, inducing the calcification of the cartilaginous matrix. At this stage, cells in the perichondrium differentiate into osteoblasts which secrete bone matrix which in turn mineralizes,

forming the bone collar around the mineralized hypertrophic cartilage of the anlage. At this point, osteoclast precursors migrate from the blood compartment to the bone surface where they mature to form osteoclasts. These osteoclasts then resorb bone and cartilage and penetrate the anlage, allowing blood vessels to penetrate the bone collar and invade the mineralized matrix, initiating the ossification process.

Thus, this developmental process could be particularly sensitive to defects in the ability of osteoclasts and their precursors to migrate. Indeed, examination of c-Cbl$^{-/-}$ metatarsals at a time when osteoclasts invade the cartilage (E17.5) (Engsig et al., 2000) demonstrates that, although the total number of osteoclasts and their precursors was unchanged, there was a significant decrease in the number of osteoclasts that had penetrated the hypertrophic cartilage and a corresponding accumulation of osteoclasts outside the outer surface of the bone collar. This clearly demonstrates that osteoclasts that lack c-Cbl exhibit a decreased ability to migrate in vivo, consistent with our findings in vitro (Sanjay et al., 2001b).

This conclusion was supported by several independent observations. First, cartilaginous remnants persist longer in the spongiosa of c-Cbl$^{-/-}$ mice than in controls. This is considered a pathognomonic sign of decreased osteoclast activity, most prominent in osteopetrosis (Sims and Baron, 2000). Second, the ossification of the epiphysis was markedly delayed in the absence of c-Cbl. This process is also known to require the penetration of osteoclasts into the hypertrophic cartilage. Third, the hypertrophic zone of the growth plate was increased in c-Cbl$^{-/-}$ mice at some stages of development. This is also observed in conditions where the resorption of calcified cartilage is decreased (Soriano et al., 1991).

We also found that c-Cbl$^{-/-}$ mice display a transient delay in growth, with a concomitant decrease in proliferating zone thickness and in total tibia length, that coincides with the onset of the delay in the formation of the secondary center of ossification and in the vascularization of the epiphysis. Although this finding may suggest that the absence of c-Cbl could have a direct, cell-autonomous effect on chondrocyte biology, it is intriguing that, in other animal models with delayed ossification and vascularization of the epiphysis, a delay in growth and a decrease in proliferation rate of the chondrocytes is observed as well (Holmbeck et al., 1999). It has been recently reported that the developing cartilaginous epiphysis is not only avascular, but is also a hypoxic tissue, and that the transcription factor hypoxia-induced factor-1α (HIF-1α) plays a crucial role in chondrocyte growth arrest and survival (Schipani et al., 2001). The absence of HIF-1α caused apoptosis of chondrocytes in the central part of the epiphysis, where hypoxia is more limiting, and increased chondrocyte proliferation in peripheral areas where oxygen and nutrients can diffuse from the perichondrium. It is therefore tantalizing to speculate that the delay in the ossification and vascularization we observed, as a consequence of a defect in the ability of osteoclasts to invade the hypertrophic cartilage, could be at the basis of the observed transient reduced growth in early postnatal life. Of course, more experiments will be necessary to clearly demonstrate this last point. Nonetheless, c-Cbl-null mice could be an useful animal model to further clarify the roles of hypoxia and of vascularization in the developing endochondral bones.

Taken together, our present results demonstrate that, in the absence of c-Cbl, osteoclast migration, and consequently function, is altered in vivo, as it is in vitro. We have shown in previous studies that c-Cbl plays a role in the rapid assembly and disassembly of adhesion structures that is required for cell motility (Sanjay et al., 2001b). This function of c-Cbl involves both downregulation of Src kinase activity (Sanjay et al., 2001b) and ubiquitination of both c-Src and c-Cbl itself in the molecular complex initially assembled following integrin engagement (Yokouchi et al., 2001). In the long run, another member of the Cbl family (probably Cbl-b, since Cbl-3, the third known mammalian Cbl protein, is not expressed in hematopoietic cells nor in bone marrow; Keane et al., 1999) is apparently capable of compensating for the absence of this function sufficiently for bone remodeling to proceed normally. Our study shows, however, that this redundancy is not fully efficient, given that cell migration is still partially impaired, albeit not enough to alter bone resorption at the tissue level.

Acknowledgments

We thank Drs. H. Gu and M. Naramura at the NIH for kindly providing c-Cbl-null mice. We also thank Lynn Neff and Jennifer Juel for skillful technical assistance, and Karen Ford for maintaining the animal colonies. This work was supported by National Institutes of Health grants AR42927 and DE04724 (R.B.).

References

Akatsu, T., Tamura, T., Takahashi, N., Udagawa, N., Tanaka, S., Sasaki, T., Yamaguchi, A., Nagata, N., Suda, T., 1992. Preparation and characterization of a mouse osteoclast-like multinucleated cell population. J. Bone Miner. Res. 7, 1297–1306.

Baron, R., Ravesloot, J.-H., Neff, L., Chakraborty, M., Chatterjee, D., Lomri, A., Horne, W., 1993. Cellular and molecular biology of the osteoclast, in: Noda, M. (Ed.), Cellular and Molecular Biology of Bone, Academic Press, San Diego, pp. 445–495.

Engsig, M.T., Chen, Q.J., Vu, T.H., Pedersen, A.C., Therkidsen, B., Lund, L.R., Henriksen, K., Lenhard, T., Foged, N.T., Werb, Z., Delaisse, J.M., 2000. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J. Cell Biol. 151, 879–889.

Galisteo, M.L., Dikic, I., Batzer, A.G., Langdon, W.Y., Schlessinger, J., 1995. Tyrosine phosphorylation of the *c-cbl* proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. J. Biol. Chem. 270, 20242–20245.

Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S.A., Mankani, M., Robey, P.G., Poole, A.R., Pidoux, I., Ward, J.M., Birkedal-Hansen, H., 1999. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell 99, 81–92.

Joazeiro, C.A.P., Wing, S.S., Huang, H.-K., Leverson, J.D., Hunter, T., Liu, Y.-C., 1999. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 286, 309–312.

Keane, M.M., Ettenberg, S.A., Nau, M.M., Banerjee, P., Cuello, M., Penninger, J., Lipkowitz, S., 1999. cbl-3: a new mammalian cbl family protein. Oncogene 18, 3365–3375.

Levkowitz, G., Waterman, H., Ettenberg, S.A., Katz, M., Tsygankov, A.Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., Yarden, Y., 1999. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol. Cell 4, 1029–1040.

Liu, Y.-C, Gu, H., 2002. Cbl and Cbl-b in T-cell regulation. Trends Immunol. 23, 140–143.

Lupher Jr., M.L., Reedquist, K.A., Miyake, S., Langdon, W.Y., Band, H., 1996. A novel phosphotyrosine-binding domain in the N-terminal transforming region of Cbl interacts directly and selectively with ZAP-70 in T cells. J. Biol. Chem. 271, 24063–24068.

Marchisio, P.C., Cirillo, D., Naldini, L., Primavera, M.V., Teti, A., Zambonin-Zallone, A., 1984. Cell-substratum interaction of cultured avian osteoclasts is mediated by specific adhesion structures. J. Cell Biol. 99, 1696–1705.

Marchisio, P.C., Cirillo, D., Teti, A., Zambonin-Zallone, A., Tarone, G., 1987. Rous sarcoma virus-transformed fibroblasts and cells of monocytic origin display a peculiar dot-like organization of cytoskeletal proteins involved in microfilament-membrane interactions. Exp. Cell Res. 169, 202–214.

McHugh, K.P., Hodivala-Dilke, K., Zheng, M.H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F.P., Hynes, R.O., Teitelbaum, S.L., 2000. Mice lacking β_3 integrins are osteosclerotic because of dysfunctional osteoclasts. J. Clin. Invest. 105, 433–440.

Meng, F., Lowell, C.A., 1998. A β_1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J. 17, 4391–4403.

Murphy, M.A., Schnall, R.G., Venter, D.J., Barnett, L., Bertoncello, I., Thien, C.B., Langdon, W.Y., Bowtell, D.D., 1998. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol. Cell. Biol. 18, 4872–4882.

Naramura, M., Jang, I.-K., Kole, H., Huang, F., Haines, D., Gu, H., 2002. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat. Immunol. 3, 1192–1199.

Naramura, M., Kole, H.K., Hu, R.J., Gu, H., 1998. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc. Natl. Acad. Sci. USA 95, 15547–15552.

Ojaniemi, M., Martin, S.S., Dolfi, F., Olefsky, J.M., Vuori, K., 1997. The proto-oncogene product $p120^{cbl}$ links c-Src and phosphatidylinositol 3′-kinase to the integrin signaling pathway. J. Biol. Chem. 272, 3780–3787.

Ota, S., Hazeki, K., Rao, N., Lupher Jr., M.L., Andoniou, C.E., Druker, B., Band, H., 2000. The RING finger domain of Cbl is essential for negative regulation of the Syk tyrosine kinase. J. Biol. Chem. 275, 414–422.

Ota, Y., Samelson, L.E., 1997. The product of the proto-oncogene c-*cbl*: a negative regulator of the Syk tyrosine kinase. Science 276, 418–420.

Parfitt, A.M., Drezner, M.K., Glorieux, F.H., Kanis, J.A., Malluche, H., Meunier, P.J., Ott, S.M., Recker, R.R., 1987. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J. Bone Miner. Res. 2, 595–610.

Sanjay, A., Horne, W.C., Baron, R., 2001a. The Cbl family: ubiquitin ligases regulating signaling by tyrosine kinases. Sci. STKE 2001, E40.

Sanjay, A., Houghton, A., Neff, L., Didomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W.C., Baron, R., 2001b. Cbl associates with Pyk2 and Src to regulate Src kinase activity, $\alpha_v\beta_3$ integrin-mediated signaling, cell adhesion, and osteoclast motility. J. Cell Biol. 152, 181–195.

Sato, T., Foged, N.T., Delaisse, J.M., 1998. The migration of purified osteoclasts through collagen is inhibited by matrix metalloproteinase inhibitors. J. Bone Miner. Res. 13, 59–66.

Scaife, R.M., Langdon, W.Y., 2000. c-Cbl localizes to actin lamellae and regulates lamellipodia formation and cell morphology. J. Cell Sci. 113, 215–226.

Schipani, E., Ryan, H.E., Didrickson, S., Kobayashi, T., Knight, M., Johnson, R.S., 2001. Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. Genes Dev. 15, 2865–2876.

Sims, N., Baron, R., 2000. Bone cells and their function, in: Canalis, E. (Ed.), Skeletal Growth Factors, Lippincott Williams and Wilkins, Philadelphia, pp. 1–16.

Sims, N.A., Clement-Lacroix, P., Da Ponte, F., Bouali, Y., Binart, N., Moriggl, R., Goffin, V., Coschigano, K., Gaillard-Kelly, M., Kopchick, J., Baron, R., Kelly, P.A., 2000. Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. J. Clin. Invest. 106, 1095–1103.

Soriano, P., Montgomery, C., Geske, R., Bradley, A., 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64, 693–702.

Stickel, S.K., Wang, Y., 1987. Alpha-actinin-containing aggregates in transformed cells are highly dynamic structures. J. Cell Biol. 104, 1521–1526.

Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J.B., Baron, R., 1996. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 383, 528–531.

Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science 289, 1504–1508.

Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W.C., Zhang, H., Yoshimura, A., Baron, R., 1999. Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. J. Biol. Chem. 274, 31707–31712.

Yokouchi, M., Kondo, T., Sanjay, A., Houghton, A., Yoshimura, A., Komiya, S., Zhang, H., Baron, R., 2001. Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. J. Biol. Chem. 276, 31185–31193.

Zambonin-Zallone, A., Teti, A., Gaboli, M., Marchisio, P.C., 1989. Beta 3 subunit of vitronectin receptor is present in osteoclast adhesion structures and not in other monocyte-macrophage derived cells. Connect. Tissue Res. 20, 143–149.

Zambonin-Zallone, A., Teti, A., Primavera, M.V., 1982. Isolated osteoclasts in primary culture: first observations on structure and survival in culture media. Anat. Embryol. 165, 405–413.

Zell, T., Warden, C.S., Chan, A.S.H., Cook, M.E., Dell, C.L., Hunt III, S.W., Shimizu, Y., 1998. Regulation of β1-integrin-mediated cell adhesion by the Cbl adaptor protein. Curr. Biol. 8, 814–822.