ORIGINAL ARTICLE

Identification and characterization of anti-osteoclastogenic peptides derived from the cytoplasmic tail of receptor activator of nuclear factor kappa B

Yuu Taguchi · Yoko Kiga · Jin Gohda · Jun-ichiro Inoue

Abstract Pathological bone resorption by osteoclasts is primarily treated with bisphosphonates. Because the administration of bisphosphonates is associated with a risk for multiple adverse symptoms, a precise understanding of the mechanisms underlying osteoclastogenesis is required to develop drugs with minimal side-effects. Osteoclastogenesis depends on receptor activator of nuclear factor kappa B (RANK) signaling mediated by TRAF6. We previously identified a *h*ighly *c*onserved domain in the cytoplasmic tail of *R*ANK (HCR), which did not share any significant homology with other proteins and was essential for osteoclastogenesis. HCR acts as a platform for the formation of Gab2- and Vav3-containing signal complexes, and ectopic expression of the HCR peptide inhibits osteoclastogenesis. Here, we uncover the mechanisms of HCR peptide-mediated inhibition of osteoclastogenesis. Expression of either the amino- or carboxyl-terminal half of the HCR peptide (N- or C-peptide) independently inhibited RANK signaling prior to cell–cell fusion. In contrast, expression of the GY-peptide, which is a part of the C-peptide, did not significantly affect prefusion RANK signaling, but did inhibit cell–cell fusion to prevent formation of multinucleated mature osteoclasts. Moreover, Gab2, which is involved in RANK signaling by binding TRAF6, bound the C-peptide but not the N-peptide, suggesting that the C- and the N-peptides sequester TRAF6 in a Gab2-dependent and Gab2-independent manner, respectively. In contrast, the GY-peptide did not bind Gab2 but could bind Vav3, which mediates signaling for cell–cell fusion. Collectively, we propose that the HCR peptide inhibits osteoclastogenesis through two modes of action— inhibition of (1) prefusion RANK signaling and (2) cell–cell fusion by blocking TRAF6- and Vav3-mediated signaling, respectively.

Keywords Osteoclast · RANK · Signal transduction · Inhibitor · Gab2

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Introduction

Osteoclasts are bone-resorbing, multinucleated cells derived from hematopoietic stem cells and play a crucial role in bone homeostasis by collaborating with osteoblasts, which are responsible for bone formation [1]. Excessive induction or activity of osteoclasts causes pathological bone resorption, as observed in postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and tumor bone metastases [1, 2]. In these pathological conditions, bisphosphonates are primarily used for treatment because the accumulation of bisphosphonates in osteoclasts, which is caused by selective absorption of bisphosphonates to the mineral surface and their subsequent internalization into osteoclasts, inhibits their bone-resorbing activity [3]. However, the administration of bisphosphonates is associated with a risk for multiple adverse symptoms including

renal toxicity [4], gastrointestinal tract disturbances [5], muscular–osseous pains [6] and mandible necrosis [7]. Moreover, although it is controversial, an increased risk of atrial fibrillation in female patients using bisphosphonates has been reported [7]. Therefore, a precise understanding of the mechanisms that regulate osteoclast formation is essential for understanding the onset of skeletal diseases and for developing drugs to treat such diseases with minimal side-effects.

Osteoclastogenesis is tightly regulated by receptor activator of nuclear factor kappa B (RANK) and its cognate ligand, RANKL [8, 9]. Upon binding of RANKL to RANK on osteoclast precursor cells, tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is recruited to the intracellular domain of RANK [10]. We and others have previously reported that TRAF6-deficient mice show severe osteopetrosis and defective osteoclast formation because of abrogated RANK signaling, indicating that TRAF6 is an essential molecule in RANK signaling [11–14]. TRAF6 then acts as an E3 ubiquitin ligase to generate unanchored Lys63-linked polyubiquitin chains or conjugate the polyubiquitin chains to transforming growth factor beta (TGF-β)-activated kinase 1 (TAK1) or TRAF6 itself, which results in the activation of TAK1 [15–17]. Activated TAK1, in turn, activates the inhibitor of kappa B (IκB) kinase (IKK) complex and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, consequently leading to the activation of NF-κB and AP-1, essential transcription factors for osteoclastogenesis [1, 18–22]. The RANK/RANKL interaction also induces phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, such as DNAX-activating protein 12 (DAP12) and Fc-receptor common γ-subunit (FcRγ). This leads to the activation of phospholipase Cγ2 (PLCγ2), which mediates calcium (Ca^{2+}) oscillation, an essential event for induction of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a master transcription factor of osteoclastogenesis [23–27]. Because signals from these ITAM-harboring adaptors are essential for NFATc1 induction but cannot independently induce osteoclastogenesis, they are considered costimulatory signals [1]. Cooperation of the costimulatory signal-mediated Ca^{2+} oscillation and the activation of NF-κB and AP-1 are required for the induction of NFATc1 [24, 26, 28]. Activation of NF-κB, MAPKs and PLCγ2 occurs during the first hour after stimulation (early-phase signals), whereas the Ca^{2+} oscillations and NFATc1 activation start approximately 12 h after stimulation, reaching a maximum at approximately 48 h after stimulation (late-phase signals) [29]. During the late phase, NFATc1 induces expression of osteoclast-specific genes, such as tartrate-resistant acid phosphatase (TRAP) [30], osteoclast-associated receptor

[31], cathepsin K [32], β3-integrin [33] and calcitonin receptor [34]. Osteoclast precursor cells are then fused to one another and differentiate into multinucleated mature osteoclasts [1].

Despite recent advances in the understanding of osteoclastogenic signaling, it is still difficult to propose any of the signaling proteins described above as a suitable target for developing drugs for pathogenic bone resorption, as these proteins are commonly used in other signaling pathways and, as such, may lead to adverse effects. However, we have identified a unique amino acid sequence in the cytoplasmic tail of RANK, termed *h*ighly *c*onserved domain in *R*ANK (HCR) (amino acid 487–546 of mouse RANK, Fig. 2a), as an essential domain for osteoclastogenesis [29]. The mutant RANK (ΔHCR) that lacks HCR but contains the TRAF6-binding site is able to activate the early-phase signals but not the late-phase signals [29]. We have also demonstrated that HCR acts as a platform for RANKL stimulation-induced formation of a signaling complex containing Gab2, PLCγ2 and TRAF6, which continuously activates NF-κB, PLCγ2 and NFATc1 to establish the late-phase signals [29, 35]. Moreover, from a therapeutic perspective, we have demonstrated that ectopic expression of the HCR peptide can inhibit RANK-induced osteoclast differentiation by blocking differentiation of TRAP$^+$ mononuclear cells [29]. Because the amino acid sequence of HCR does not show any homology to other proteins, the HCR peptide may work as a specific inhibitor of osteoclastogenesis with minimal side-effects. Kim et al. [36] also reported that a peptide containing a portion of HCR, designated as RANK receptor inhibitor (RRI) (Fig. 2a), inhibits osteoclastogenesis. However, in contrast to our report, they showed that the RRI expression did not affect prefusion events, including differentiation of TRAP$^+$ mononuclear cells, but blocked cell–cell fusion, which generates multinucleated bone-absorbing TRAP$^+$ cells [36]. With the idea of applying the HCR peptide as an anti-osteoclastogenic drug, we further analyzed the molecular mechanisms of HCR peptide-mediated inhibition of osteoclastogenesis to explain the discrepancy between the two groups. We found that the HCR peptide has a dual mode of action in blocking osteoclast formation.

Materials and methods

Antibodies and plasmids

The following antibodies were used: antibodies against phospho-IκBα, phospho-JNK, phospho-ERK or phospho-p38 (Cell Signaling Technology, Danvers, MA, USA); NFATc1, α-tubulin, glutathione S-transferase (GST), c-Myc, HA and p38 (Santa Cruz Biotechnology, Santa

Cruz, CA, USA); FLAG (Sigma-Aldrich, St. Louis, MO, USA). The construction of a retroviral vector expressing FLAG-tagged HCR (pMXs-FLAG-HCR) was previously described [29]. To generate pMXs-TAP-HCR, a sequence encoding a FLAG-tag and tandem Strep-tag II was inserted upstream of the FLAG-tag in pMXs-FLAG-HCR, thereby generating FLAG–Strep–Strep–FLAG-HCR (TAP-HCR). In addition, retroviral vectors of HCR derivatives, including pMXs-TAP-N, pMXs-TAP-C and pMXs-TAP-GY, were constructed by performing inverse-PCR with the appropriate primers [37, 38]. The retroviral vector pMXs-TAP expressing only TAP-tag (FLAG–Strep–Strep–FLAG) was constructed by inserting the coding sequence of the TAP-tag into pMXs-puro [39]. To generate expression vectors of GST–HCR and GST–HCR derivatives, the coding sequences of HCR and HCR derivatives were transferred from pMXs-TAP-HCR (and its derivatives) to the pMEG2 vector [40], which encodes a GST-tag. The construction of a retroviral vector expressing Myc-tagged Gab2 (pMXs-Gab2-Myc) and that of pRK5-Gab2-Myc were previously described [29]. pMXs-HA-TRAF6 was constructed by inserting the coding sequence of the HA-tag into pMXs-TRAF6 [21], and pRK5-FLAG-Vav3 was constructed by inserting the coding sequence of Vav3 amplified by PCR into pRK5 vector [29].

Retrovirus-mediated gene transfer and ex vivo assays for osteoclastogenesis

The packaging cell line Plat-E (2×10^6 cells) was transfected with 5 μg of various retroviral vectors. Virus stocks were prepared by collecting culture media 48 h after transfection. Bone marrow cells derived from C57BL/6 mice were cultured on 12- or 48-well plate for 12 h in α-MEM containing 10 % fetal bovine serum supplemented with culture medium from CMG14-12 cells as a source of M-CSF [41]. Bone marrow cells were then incubated in virus stock medium containing 10 μg/ml of polybrene (Sigma-Aldrich) for 6 h and further cultured for 1 day in the presence of M-CSF and puromycin to select infected cells. Cells were then stimulated with the indicated concentrations of RANKL (Wako, Osaka, Japan). Adherent cells were fixed in 4 % paraformaldehyde, treated with ethanol/acetone (50:50 v/v) and stained for TRAP [21]. TRAP$^+$ multinucleated cells containing more than five nuclei were classified as osteoclasts.

Glutathione S-transferase pulldown and immunoblot analysis

For the preparation of whole cell lysates, cells were washed once with phosphate buffered saline (PBS) and lysed in the sample buffer [67.5 mM Tris–HCl (pH 7.2),

2.25 % SDS, 10 % glycerol, 5 % β-mercaptoethanol]. For GST pulldown, cells were lysed in TNE buffer [20 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate] and centrifuged to remove cellular debris. The resulting supernatant was mixed with Glutathione Sepharose^{TM} 4B (GE Healthcare, Buckinghamshire, UK). Sepharose beads were washed five times with TNE buffer and then mixed with the sample buffer. For immunoblot analysis, the GST pulldown samples and the whole cell lysates were separated on polyacrylamide/SDS gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with the appropriate primary antibodies. Immunoreactive proteins were visualized with anti-rabbit or anti-mouse immunoglobulin (Ig) G conjugated to horseradish peroxidase (GE Healthcare) using an ECL detection system (GE Healthcare).

Immunostaining and microscopic analysis

Bone marrow cells were seeded on 48-well plates and cultured for 1 day in medium containing M-CSF. Cells were then infected with retrovirus expressing HCR or HCR derivative peptides and stimulated with RANKL. Cells were fixed with 4 % paraformaldehyde in PBS, treated with ethanol/acetone (50:50 v/v) and stained for TRAP. The stained cells were then incubated with an anti-FLAG antibody (Sigma-Aldrich) for 1 h and further incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 1 h. Samples were observed using a fluorescence microscope (IX71; OLYMPUS, Tokyo, Japan).

Results

The HCR peptide can inhibit osteoclastogenesis by blocking both the early and the late phases of RANK signaling

Bone marrow-derived macrophages (BMMs) infected with the retrovirus (F-HCR virus) expressing both the N-terminal-FLAG-tagged HCR peptide (FLAG-HCR) and the puromycin resistance gene (puromycin N-acetyl-transferase; *pac*) and BMMs infected with control virus only expressing *pac* were treated with puromycin to select for infected cells, followed by stimulation with RANKL for 3 days. The FLAG-HCR peptide-expressing cells failed to form multinucleated TRAP$^+$ osteoclasts, whereas control virus-infected cells efficiently differentiated into osteoclasts (Fig. 1a), indicating that ectopic expression of the HCR peptide inhibits osteoclastogenesis. This result

confirms our previous observations that were based on different experimental conditions [29].

To elucidate the molecular mechanism of the HCR peptide-mediated inhibition of osteoclastogenesis, we investigated the NFATc1 expression and NF-κB activation in the late phase because HCR, when acting as a part of RANK, is dispensable for the early phase of RANK signaling but essential for their late-phase signals [29]. BMMs infected with the control or F-HCR virus were stimulated with RANKL for up to 48 h. Cell lysates were then prepared at various times after stimulation and subjected to

immunoblot analysis. NFATc1 protein levels and phosphorylated IκBα were markedly decreased in the HCR peptide-expressing cells compared to control virus-infected cells (Fig. 1b), indicating that the HCR peptide can inhibit the sustained activation of RANK signaling and lead to NFATc1 induction in the late phase, as we expected. However, when we analyzed the early-phase signals, the amounts of the phosphorylated forms of IκBα, JNK, ERK and p38 in the HCR peptide-expressing cells unexpectedly decreased compared to control virus-infected cells (Fig. 1c). These results indicate that the ectopic expression
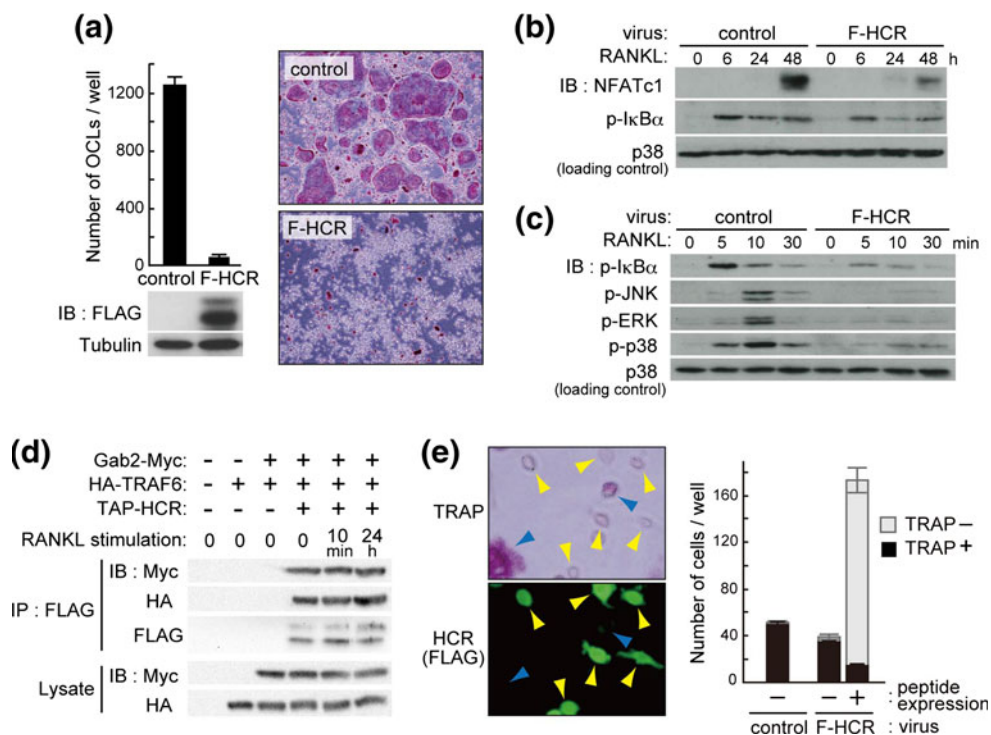

Fig. 1 Ectopic expression of the HCR peptide attenuates the early and late phases of RANK signaling and blocks osteoclastogenesis. a Expression of the HCR peptide blocks osteoclastogenesis. BMMs were cultured in a 12-well plate for one day with M-CSF and infected with the control retrovirus or retrovirus expressing F-HCR peptides. Peptide expression was confirmed by immunoblot analysis (*lower left*). Infected cells were stimulated with 100 ng/ml RANKL for 3 days, then fixed with formaldehyde and stained with TRAP. TRAP$^+$ multinucleated cells containing >5 nuclei were classified as osteoclasts (*upper left*). Representative images are shown on the right. b Attenuation of the late phase of RANK signaling by the HCR peptide. BMMs were cultured and treated as described in a. Cells were then stimulated with 100 ng/ml RANKL for the indicated periods of time. Whole cell lysates were prepared, and immunoblot analysis was carried out using antibodies against NFATc1, phosphorylated IκBα (*p-IκBα*) and p38. c Attenuation of the early phase of RANK signaling by the HCR peptide. Experiments were performed as described in b except that immunoblot analysis was carried out using antibodies against p-IκBα, phosphorylated JNK (*p-JNK*), phosphorylated ERK (*p-ERK*), phosphorylated p38 (*p-p38*) and p38. d Binding analysis of Gab2 and TRAF6 to the HCR peptide. BMMs were infected with retrovirus expressing Gab2-Myc, HA-TRAF6 and

F-HCR. Cell lysate in TNE buffer were prepared after stimulation with 100 ng/ml RANKL, and immunoprecipitation assays were performed with anti-FLAG antibody. The precipitates were subjected to immunoblot analysis. e The cell-autonomous role of the HCR peptide. BMMs were cultured in a 48-well plate and infected with control retrovirus or retrovirus expressing F-HCR peptides. Cells were stimulated with 100 ng/ml RANKL for 3 days, then fixed with formaldehyde and stained with TRAP. Cells were further immunostained using anti-FLAG and Alexa Fluor 488 goat anti-mouse IgG to visualize the F-HCR peptide. Representative images of cells infected with retrovirus expressing F-HCR peptides are shown on the *left*. Statistic analyses of numbers of mononuclear and multinuclear cells that are peptide$^+$TRAP$^+$, peptide$^+$TRAP$^-$, peptide$^-$TRAP$^+$, or peptide$^-$TRAP$^-$ are shown on the on the *right*. The *yellow arrows* indicate cells expressing high levels of F-HCR peptides (peptide expression '+' in the *right panel*), and the *blue arrows* indicate cells with low or undetectable levels of F-HCR peptides (peptide expression '−' in the *right panel*). The results shown in panels a–e are representative of three independent experiments except that the results shown in the *left panel* of a and the *right panel* of e indicate the mean ± SD of triplicate determinations and are representatives of two independent experiments
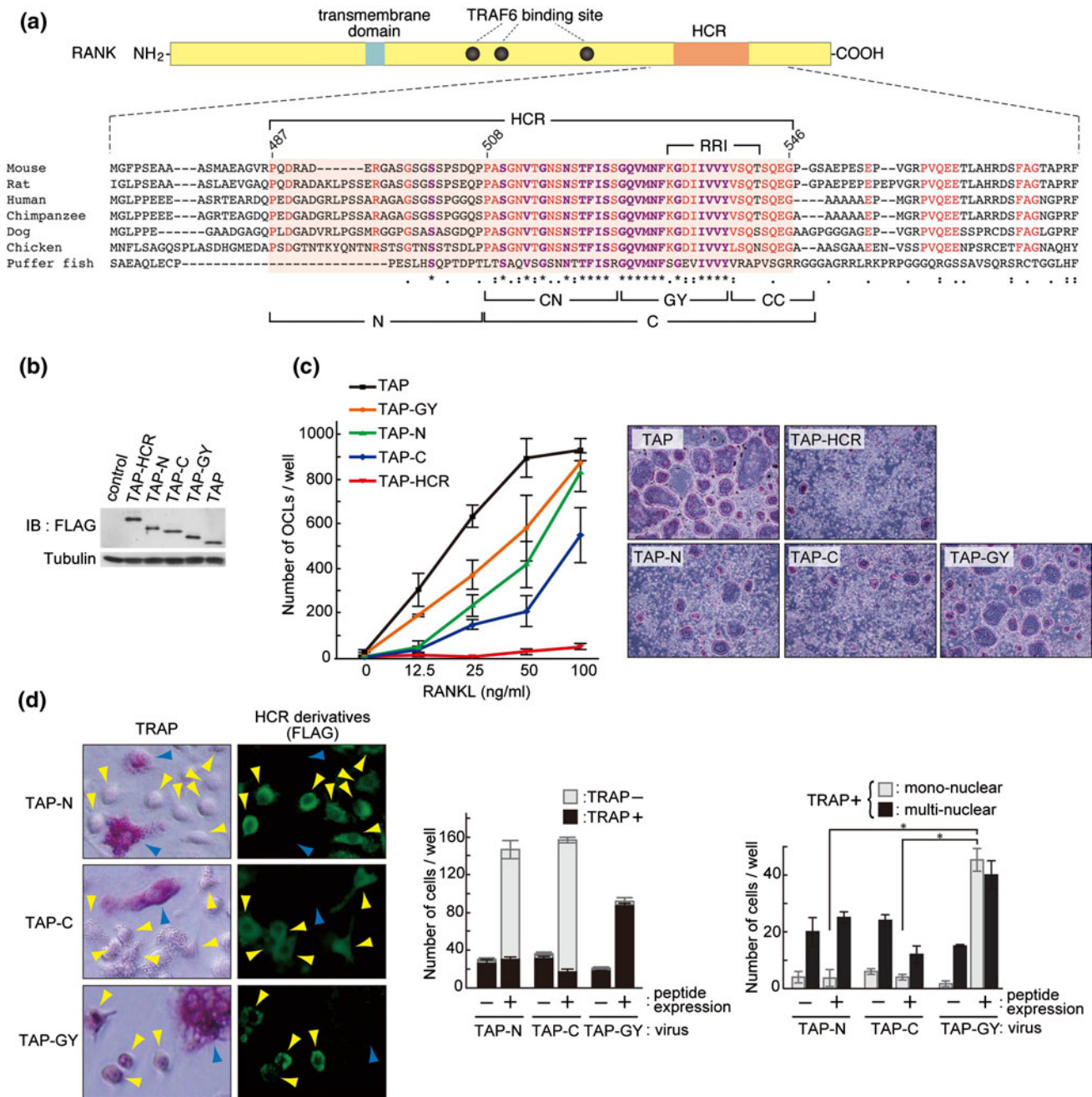
of the HCR peptide not only inhibits the late phase, but also the early phase of RANK signaling. This discrepancy may be explained by the possible sequestering of TRAF6 by the HCR peptide through Gab2, as Gab2 is able to bind both TRAF6 and the HCR peptide [29]. To test this hypothesis, we checked whether the HCR peptide could capture TRAF6. BMMs were infected with retrovirus expressing Myc-tagged Gab2 (Gab2-Myc), HA-tagged TRAF6 (HA-TRAF6) and FLAG-HCR, and cell lysates were prepared in the presence or absence of RANKL stimulation. Immunoprecipitation assay was performed with the anti-FLAG antibody and the precipitated Gab2-Myc and HA-TRAF6

were visualized by immunoblotting. Gab2-Myc and HA-TRAF6 were co-precipitated with the HCR peptide irrespective of RANKL-stimulation (Fig. 1d), indicating that the HCR peptide has the ability to sequester both Gab2 and TRAF6 in BMMs.

Since the expression levels of the HCR peptide and the extent of differentiation as assessed by TRAP staining varied among infected cells within a single culture well, we then analyzed the correlation between these two factors. BMMs were infected with the F-HCR virus or control virus, and cultured in medium containing puromycin and RANKL for 3 days. Cells were then stained for TRAP and

◄ **Fig. 2** Comparisons of the inhibitory abilities of various HCR derivative peptides on osteoclastogenesis. **a** Alignment of the amino acid sequences of RANK from various vertebrates. A schematic diagram of RANK is shown (*upper*). The amino acid sequences of RANK in mouse (NP_033425), rat (XP_573424), human (NP_003830), chimpanzee (XP_523949), dog (XP_541077), chicken (XP_001233219) and pufferfish (CAF97951) were aligned with CLUSTALW (version 1.83) (*lower*) [54]. *Numbers* indicate the amino acid position from the N-terminus in the mouse sequence. Amino acids that were identical among the six species, except for pufferfish, are shown in *red*, and those that were identical among all seven species are shown in *purple*. HCR, N-, C-, GY-, CN-, CC-region, and the region corresponding to RRI-peptides in RANK are indicated. **b** Expression levels of various HCR derivative peptides. BMMs were infected with retroviruses expressing TAP-tag, TAP-H-, TAP-N-, TAP-C- or TAP-GY-peptides. Cells were then cultured for 1 day. Cell lysates were prepared and subjected to immunoblot analysis using anti-FLAG and anti-α-tubulin antibodies. **c** Inhibition of osteoclastogenesis by various HCR derivative peptides. BMMs were cultured in a 12-well plate and treated as described in **b**. Cells were stimulated with 12.5, 25, 50 or 100 ng/ml RANKL for 3 days, then fixed with formaldehyde and stained with TRAP. TRAP$^+$ multinucleated cells containing >5 nuclei were classified as osteoclasts and were counted (*left*). Representative images of osteoclastogenesis at 25 ng/ml RANKL stimulation in the presence of various HCR derivative peptides are shown (*right*). **d** Two distinct modes of inhibition of osteoclastogenesis by the HCR derivative peptides. BMMs were cultured in a 48-well plate and treated as described in **b**. Cells were stimulated with 25 ng/ml RANKL for 3 days, then fixed with formaldehyde and stained with TRAP. Cells were further immunostained using anti-FLAG and Alexa Fluor 488 goat anti-mouse IgG to visualize the HCR derivative peptides. The *yellow arrows* indicate cells expressing high levels of TAP-HCR derivative peptides (peptide expression '+' in the *middle* and the *right panels*), and the *blue arrows* indicate cells with low or undetectable levels of TAP-HCR derivative peptides (peptide expression '−' in the *middle* and the *right panels*). In the *middle panel*, numbers of TRAP$^-$ mononuclear cells and those of TRAP$^+$ cells including both mononuclear and multinuclear cells are statistically analyzed. In the *right panel*, numbers of mononuclear TRAP$^+$ cells and those of multinuclear TRAP$^+$ cells are shown separately. The results shown in **b**, the *right panel* of **c** and the *left panel* of **d** are representative of three independent experiments. The results shown in the *left panel* of **c**, and the *middle* and the *right panels* of **d** indicate the mean ± SD of triplicate determinations of two independent experiments. *$P < 0.01$ (Student's *t* test)

immunostained with the anti-FLAG antibody to visualize HCR peptide expression levels. Cells highly expressing F-HCR peptides (Fig. 1e, left, yellow arrows) expressed little or almost no TRAP, while most cells expressing little or no F-HCR peptide (Fig. 1e, left, blue arrows) were positive for TRAP-staining (Fig. 1e, right). These results strongly suggest that HCR peptides inhibit osteoclastogenic signals in a cell-autonomous manner, without the involvement of secreted factors that act in trans to inhibit osteoclastogenesis of other cells.

It has been reported that the expression of the RRI peptide (KGDIIVVYVSQT, Fig. 2a) resulted in blocking cell–cell fusion, but not differentiation of TRAP$^+$ mononuclear monocytes [36], indicating that the RRI peptide inhibits neither the early nor late phases of RANK

signaling. Given that the expression of the HCR peptide inhibits prefusion osteoclastogenic signaling, including both the early and late phases of RANK signaling, the amino acid sequence of the HCR excluding the RRI peptide may mediate the inhibition of RANK signaling (Fig. 1b, c). Therefore, the HCR peptide could inhibit osteoclastogenesis through at least two different modes of action—inhibition of prefusion signal transduction emanating from RANK and ITAM-harboring adaptors and inhibition of cell–cell fusion.

Both the N- and C-terminal regions of the HCR peptide can inhibit osteoclastogenesis

To further elucidate the molecular mechanism by which the HCR peptide mediates the inhibition of osteoclastogenesis and to identify a minimum sequence of the HCR peptide required for blocking osteoclastogenesis, we performed a domain analysis of the HCR peptide. From a therapeutic perspective, peptide length is important because smaller peptides can more efficiently enter into cells after fusion with various cell-penetrating peptides, such as the TAT-peptide and the oligo-arginine peptide [42–44].

Based on the levels of amino acid sequence conservation, HCR peptides can be divided into two subdomains—the less-conserved N-terminal region (N-region, aa 487–507) and the highly conserved C-terminal region (C-region, aa 508–548) (Fig. 2a). We thus constructed retroviral vectors expressing peptides corresponding to the N-region (N) or C-region (C) of HCR. Within the C-region, TFIS (aa 520–523), GQVMNF (aa 525–530) and IVVY (aa 535–538) were identical among various species including pufferfish (purple letters in Fig. 2a). As it has been reported that the GQVMNF and the IVVY sequences, but not TFIS, are essential for RANK-induced osteoclastogenesis [45], we also constructed a retroviral vector expressing peptides corresponding to aa 525–538 (GY-region), which covers both the GQVMNF and IVVY regions (Fig. 2a). To easily compare the relative expression levels of HCR and its derivative peptides (including N, C and GY), these peptides were each fused at their N-termini with a peptide that includes two FLAG and two Strep tags (FLAG–Strep–Strep–FLAG), designated as TAP-tag, which can be used for tandem affinity purification [46].

To test whether the HCR derivative peptides can inhibit osteoclastogenesis, BMMs were infected with control retrovirus or retrovirus expressing the TAP-tag, TAP-HCR, TAP-N, TAP-C or TAP-GY peptides. Cells were then treated with puromycin for selection of infected cells. Aliquots of cells were harvested from each sample, and total cell lysates were prepared and analyzed by immunoblot using an anti-FLAG antibody. Expression levels of various TAP-tagged peptides were nearly equal (Fig. 2b). Cells were then stimulated with RANKL at various concentrations for 3 days, and the

numbers of multinucleated TRAP$^+$ osteoclasts were counted. Cells expressing the TAP-tag formed approximately the same number of osteoclasts as mock-infected cells (Supplementary Fig. S1), indicating that the TAP-tag peptide itself does not affect osteoclastogenesis. In our in vitro differentiation system, the minimum RANKL concentration that achieved the maximum formation of osteoclasts was approximately 50 ng/ml (Fig. 2c). Therefore, the inhibitory activities of various peptides were first compared when cells were stimulated with 25 ng/ml RANKL. Ectopic expression of the TAP-GY, TAP-N or TAP-C peptides resulted in 40 %, 60 % or 75 % reduction in osteoclast formation, respectively, when compared with osteoclast formation of cells expressing the TAP-tag, whereas the expression of whole HCR peptides (TAP-HCR) completely abolished osteoclastogenesis (Fig. 2c, left, representative views of osteoclastogenesis are shown in the *right panel*). Upon RANKL stimulation at 100 ng/ml, expression of TAP-N or TAP-GY peptides barely inhibited osteoclastogenesis, whereas cells expressing the TAP-C peptide formed approximately 60 % of osteoclasts compared to those expressing the TAP-tag (Fig. 2c, left). These results indicate that TAP-C is the most potent among the HCR derivative peptides, but TAP-N also significantly inhibits osteoclastogenesis, which is unexpected because the N-region is much less conserved than the C-region. It is also interesting that the TAP-GY peptide significantly inhibits osteoclastogenesis even though it is only 14 residues long. Because osteoclastogenesis of TAP-HCR-expressing cells was completely blocked even when cells were stimulated with 100 ng/ml of RANKL, the TAP-HCR peptides were much more active than any of the other HCR derivatives in inhibiting osteoclast formation.

To test whether the HCR derivative peptides inhibit osteoclastogenic signals in a cell-autonomous manner and to understand which step of osteoclastogenic differentiation is blocked by these peptides, we analyzed correlation between the expression levels of the HCR derivative peptides and the extents of differentiation, as judged by TRAP staining and mono/multinucleated phenotype (Fig. 2d). For cells expressing the TAP-N or TAP-C peptides, >80 % of the cells highly expressing the HCR derivative peptides (Fig. 2d, left, yellow arrows) did not express TRAP, while most of the cells expressing little or no HCR derivative peptides (Fig. 2d, left, blue arrows) were positive for TRAP (Fig. 2d, middle) and had multiple nuclei (Fig. 2d, right). These results strongly suggest that the TAP-N and TAP-C peptides inhibit prefusion osteoclastogenic signals in a cell-autonomous manner. In contrast, most of the cells highly expressing the TAP-GY peptides (Fig. 2d, left, yellow arrows) expressed TRAP, and most of the cells expressing little or no TAP-GY peptide (Fig. 2d, left, blue arrows) were also positive for TRAP staining (Fig. 2d, middle). More importantly, more than half of the TRAP$^+$ cells that highly expressed the GY-peptide were mononuclear while most of the TRAP$^+$ cells that expressed low or no TAP-GY peptide in the same culture well were multinuclear (Fig. 2d, right). These results strongly suggest that the expression of the TAP-GY peptide does not inhibit prefusion events but inhibits cell–cell fusion in a cell-autonomous manner. These results are consistent with the finding by Kim et al. [36] who demonstrated that the RRI peptide, which covers a sequence similar to the TAP-GY-peptide, inhibits cell–cell fusion.

The C-region but not the N-region of the HCR peptide binds to Gab2

We then analyzed the effects of the ectopic expression of the HCR derivative peptides on RANKL-induced signaling pathways. BMMs were infected with control retrovirus or retrovirus expressing various TAP-tagged HCR derivative peptides and then treated with puromycin to select for infected cells. Aliquots of cells were harvested from each sample to confirm that the expression levels of various TAP-tagged peptides were nearly equal (Fig. 2b). Cells were then stimulated with 25 ng/ml RANKL for the indicated times, and cell lysates were prepared and subjected to immunoblot analysis. Under the condition where the expression of the TAP-tag itself had little effect on the early and late phases of RANK signaling (Supplementary Figs. S2, S3), the expression of TAP-N and TAP-C markedly inhibited both the late and the early phases of RANK signaling (Fig. 3a, b). The expression of TAP-GY also inhibited the two phases of RANK signaling, although the extent of inhibition was significantly less than that observed with the N- and C-peptides (Fig. 3a, b), which is consistent with the finding by Kim et al. [36] that the RRI peptide did not inhibit RANK-induced activation of NF-κB and MAPKs prior to cell–cell fusion.

We have demonstrated that the HCR in RANK acts as a platform for the formation of Gab2-containing signal complexes, which is essential for generating a sustained RANK signal and inducing NFATc1 expression during osteoclastogenesis [29, 35]. Moreover, it has been reported that Vav3, a guanine nucleotide exchange factor, interacts with the IVVY sequence located in the HCR, and that Vav3 is involved in cell–cell fusion and actin ring formation by transducing small GTPase signaling in BMMs and mature osteoclasts [36]. To understand the molecular mechanisms by which the non-overlapping N- and C-peptides can independently inhibit prefusion RANK signaling and why the GY-peptide, a part of the C-peptide, does not inhibit prefusion events, we investigated the abilities of the HCR derivative peptides to bind to Gab2 and Vav3. The C-terminal-Myc-tagged Gab2 (Gab2-Myc) or N-terminal-FLAG-tagged Vav3 (FLAG-Vav3) and HCR or HCR

derivative peptides fused to GST were coexpressed in HEK293T cells. Cell lysates were then prepared and incubated with Glutathione Sepharose 4B. GST pulldown assays were performed, and Gab2-Myc or FLAG-Vav3 bound to GST proteins was visualized by immunoblotting. FLAG-Vav3 was detected when pulled down with the C-peptide or GY-peptide as well as with the full-length HCR, whereas the N-peptide could not pull down Vav3 (Fig. 3c), indicating that Vav3 interacts with the GY-peptide of the HCR. Gab2-Myc was detected when pulled down with the C-peptide as well as with full-length HCR, whereas neither the N- nor GY-peptides were able to pull it down (Fig. 3d). Since the IVVY motif in the GY peptide is required for Gab2 to bind HCR [29], we further searched for the domains essential for the Gab2-HCR interaction. We generated expression vectors for two GST fusion

proteins—GST protein fused to the N-terminal half of the C-region (CN-region, aa 508–524) along with the GY-region (GST-CN-GY), and GST protein fused to the C-terminal half of C-region (CC-region, aa 539–548), again including the GY-region (GST-GY-CC) (Fig. 2a). GST pulldown assays revealed that Gab2 bound to the CN-GY-peptide but not to the GY-CC-peptide, indicating that the CN-region works as a binding site to Gab2 cooperatively with the GY-region.

Discussion

In this study, we analyzed HCR peptide domains to understand the molecular mechanisms of HCR peptide-mediated inhibition of osteoclastogenesis and found three
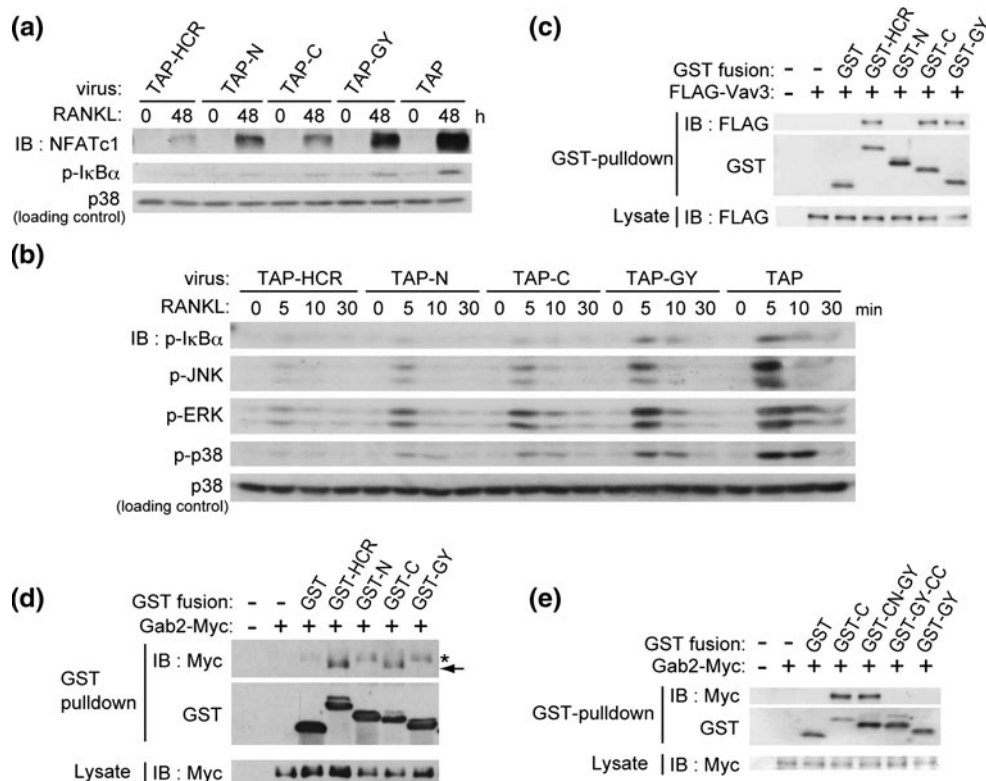

Fig. 3 Comparisons of the inhibitory abilities of various HCR derivative peptides on osteoclastogenic signaling. **a** Effect of ectopic expression of various HCR derivative peptides on the late phase of RANK signaling. BMMs were infected with retrovirus expressing TAP-tag, TAP-H-, TAP-N-, TAP-C- or TAP-GY-peptides. Cells were then stimulated with 25 ng/ml RANKL for the indicated periods of time. Whole cell lysates were prepared, and immunoblot analysis was carried out using antibodies against NFATc1, p-IκBα and p38. **b** Effect of ectopic expression of various HCR derivative peptides on the early phase of RANK signaling. Experiments were performed as described in **a** except that immunoblot analysis was carried out using antibodies against p-IκBα, p-JNK, p-ERK, p-p38 and p38. **c** Binding analysis of Vav3 to various HCR derivative peptides. HEK293T cells were transfected with plasmids expressing FLAG-Vav3 and HCR or

HCR derivative peptides fused to GST, and cells were lysed in TNE buffer. The GST pulldown assays were performed, and precipitates were subjected to immunoblot analysis. **d** Binding analysis of Gab2 to various HCR derivative peptides. Experiments were performed as described in **c** except that cells were transfected with plasmids expressing Gab2-Myc and HCR or HCR derivative peptides fused to GST. The *arrow* indicates Gab2-Myc; the *asterisk* indicates nonspecific bands. **e** Binding analysis of Gab2 to various fragments of the C-peptide. Experiments were performed as described in **d** except that cells were transfected with plasmids expressing Gab2-Myc and various fragments of the C-peptides (C-, CN-GY, GY-CC or GY-peptide) fused to GST. The results shown in panels **a–e** are representative of three independent experiments

functionally distinct regions, the N-, the C- and the GY-peptides, all of which inhibit osteoclastogenesis by their ectopic expression. Expression of the N- and C-peptides can independently block the early and late phases of pre-fusion RANK signaling, including activation of NF-κB, MAPKs and NFATc1 (Fig. 3a, b). Therefore, cells expressing the N- or C-peptides do not differentiate into TRAP+ mononuclear cells (Fig. 2c, d). In contrast, expression of the GY-peptide, which is a part of the C-peptide, slightly attenuates the early and late phases of RANK signaling (Fig. 3a, b) but does not prevent differentiation of TRAP+ mononuclear cells. More importantly, GY-peptide expression can inhibit cell–cell fusion to generate TRAP+ multinuclear cells (Fig. 2d).

Vav3 has been reported to bind to RANK through the IVVY sequence, and RANKL stimulation induces tyrosine phosphorylation of Vav3 [36]. This, in turn, activates small GTPases, such as Rac1 and Cdc42, and leads to actin reorganization [36]. Moreover, transduction of the RRI peptide, which includes the IVVY sequence, into BMMs and mature osteoclasts inhibits the activation of these small GTPases by sequestering Vav3, leading to the inhibition of both cell–cell fusion and actin ring formation [36]. Because the GY-peptide covers a sequence similar to the RRI peptide, and GY-peptide binds to Vav3 (Fig. 3c), inhibition of osteoclastogenesis by expression of the GY-peptide is likely to be due to the inhibition of the Vav3-small GTPase pathways through sequestering of the Vav3-containing signal complex (Fig. 4). Actually, the expression of GY-peptide in BMMs results in inhibition of cell–cell fusion in osteoclastogenesis (Fig. 2d), but does not significantly affect the induction of TRAP gene expression (Fig. 2d). Because the amount of phosphorylated IκBα and the expression level of NFATc1 induced by RANKL stimulation were slightly decreased compared to control (Fig. 3a, b), a threshold level of NFATc1 activation could be required for efficient cell–cell fusion. In addition to Vav3, we have demonstrated that Gab2, which plays an important role in RANK-induced osteoclastogenic signals [35], binds to the HCR peptide in an IVVY sequence-dependent manner because Gab2 does not bind to the mutant HCR peptide in which the IVVY has been replaced by LAAF [29]. Interestingly, we show that Gab2 binds the C-peptide but not the GY-peptide, even though the GY-peptide is part of the C-peptide and harbors the IVVY sequence. However, we also show that Gab2 binds the CN-GY-peptide, which is a fusion peptide consisting of the C-peptide's N-terminal half and the GY-peptide (Fig. 3e). These results indicate that the binding of Gab2 to HCR requires not only the portion of the GY-peptide that includes IVVY but also the N-terminal half of the C-region (CN-region) (Fig. 4).

Although the N-peptide does not bind to Gab2 and Vav3 (Fig. 3c, d) and is much less conserved than the C-peptide
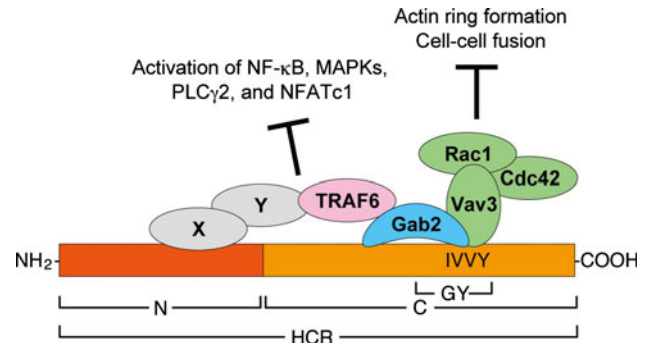

Fig. 4 A schematic model illustrating two distinct modes of inhibition of osteoclastogenesis by HCR peptides. Sequestration of Gab2-containing signal complexes by both the N- and C- peptides inhibit prefusion osteoclastogenic signals, whereas sequestration of the Vav3-containing complex by the GY-peptide inhibits cell–cell fusion and actin ring formation. See the text for details

(Fig. 2a), expression of the N-peptide unexpectedly blocked osteoclastogenesis efficiently (Fig. 2c, d). The absence of Gab2 resulted in a partial reduction of osteoclast formation [35], whereas HCR-deficient RANK completely failed to induce osteoclastogenesis [29]. This indicates that other factors, which may be components of the Gab2-containing complex, could bind to HCR to emanate osteoclastogenic signals. If some of these factors (designated as X in Fig. 4) bind to the N-peptide, expression of the N-peptide could result in the sequestration of components of the Gab2-containing complexes (designated as X and Y in Fig. 4), leading to an inhibition of the early and late phases of RANK signaling.

From a therapeutic perspective, the HCR in RANK could be useful as a therapeutic target for the treatment of pathological bone resorption, as our search of the GenBank database did not identify any proteins homologous to the primary structure of HCR (data not shown). Moreover, it is possible that either HCR or HCR derivative peptides might affect only osteoclastogenesis but not other functions of RANK signaling. RANK has been reported to be an important molecule not only in osteoclastogenesis but also in lymph node development [8], fever regulation [47], thymus organogenesis [48], mammary gland development [49] and activation of dendritic cells (DC) [50], suggesting that therapeutic methods targeting the RANK/RANKL interaction, such as RANK-Fc, may have many side-effects. However, it is known that the stimulation of DCs by the CD40 ligand (CD40L) or RANKL can activate the production of various cytokines [51]. CD40, a member of the TNF-receptor superfamily, can activate the NF-κB and MAPK pathways through TRAF6, but cannot induce osteoclastogenesis due to the lack of HCR [29]. This suggests that HCR is not involved in the activation of cytokine production in DCs even upon RANKL stimulation [21, 52, 53]. Thus, expression of the HCR derivative peptides may

not inhibit the RANKL-induced production of cytokines from DCs and other functions of RANK signaling except during osteoclastogenesis. This hypothesis is partially supported by a study showing that the RRI peptide did not inhibit production of cytokines from DCs upon RANKL stimulation [36]. Further investigation of the HCR peptide-mediated inhibition of osteoclastogenesis is required to develop therapeutic drugs aimed at inhibiting osteoclastogenic signals with the goal of treating pathological bone resorption with minimum adverse effects.

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