Dominant negative effect of the extracellular domain of CASR

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Abstract: The role of the calcium-sensing receptor (CASR) in the skeleton is unclear. CASR null mice (CASR<sup>−/−</sup>), created by deletion of exon 5 and disruption of the extracellular domain (ED), have no bone phenotype after rescue of hyperparathyroidism. In contrast, the conditional deletion in osteoblasts of exon 7 (CASR<sup>∆exon7</sup>), which encodes the 7-transmembrane and C-terminal domains, results in severe skeletal abnormalities. The disparities between these models could be explained by a hypomorphic mutation in CASR<sup>−/−</sup> mice or by the secretion of a dominant negative ED by CASR<sup>∆exon7</sup> mice. A hypomorphic mutation seems unlikely, since deletion of exon 5 disrupts CASR function <i>in vitro</i> and results in a prenatal lethal phenotype <i>in vivo</i>. We tested the alternative possibility that the ED of CASR acts as dominant negative secreted protein. Transfection of CASR<sub>1-464</sub> (encoding exons 2–5) cDNA into HEK293 cells resulted in the secretion of the predicted N-terminal CASR protein. Recombinant CASR<sub>1-464</sub> resulted in a dose-dependent and specific inhibition of extracellular calcium-mediated CASR signaling <i>in vitro</i> and increased serum PTH levels <i>in vivo</i>. These results suggest that the extracellular domain of CASR has biological activity <i>in vitro</i> and <i>in vivo</i> and may contribute to off target effects in conditional CASR<sup>∆exon7</sup> mice.

Keywords: calcium-sensing receptor, bone, GPCR, extracellular domain, dominant negative mutation

The calcium-sensing receptor (CASR) belongs to class C of the G protein-coupled receptor (GPCR) receptors, which includes metabotropic glutamate receptors, γ-aminobutyric acid type B receptors, pheromone receptors, and several orphan GPCRs. CASR has a large (>600 residues in the CASR) extracellular amino-terminal domain (ED) encoded by exons 1–6 that is required for dimerization and ligand binding and a 7-transmembrane (7TM) and a C-terminal domains encoded by exon 7. Extracellular calcium is the physiological ligand, but a diverse group of agonists can activate CASR, including amino acids, magnesium, gadolinium, neomycin, and the phenylalkylamine-derived calcimimetics. Three serine residues (Ser-170, Ser-147, and Ser-169) in the extracellular domain and a proline residue (Pro-823) in the 7TM domain of CASR are necessary for full responsiveness to extracellular calcium. CASR is widely expressed in various tissues including parathyroid gland, thyroid gland, kidney, gastrointestinal tracts, placenta, breast, skin, brain, pituitary gland, bone marrow, peripheral blood, and lens epithelial cells. In fact, using sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, CASR is reported to be present in most tissues examined to date, although many tissues express the transcript...
in low abundance. Knowledge of the biological function of CASR is largely derived from natural occurring mutations in humans and animals and engineered mutations and deletions in mouse model, which support an essential and non-redundant role of CASR to regulate calcium homeostasis, with the principal targets consisting of the parathyroid gland to regulate parathyroid hormone (PTH) and the distal tubule to regulate calcium handling by the kidneys. Inactivating mutations of one CASR allele causes familial hypocalciuric hypercalcemia and both alleles caused neonatal severe hyperparathyroidism in humans, whereas deletion of exon 5 of CASR in mice results in comparable phenotypes, characterized by the development of hypocalciuric hyperparathyroidism in the heterozygous CASR+/− mice and severe hypercalcemia and hyperparathyroidism in the CASR null mice. In contrast, spontaneously occurring activating mutations of CASR results in hypoparathyroidism in both humans and mice. Additional studies of CASR−/− mice lacking exon 5 have implicated a role of CASR in regulating PTH-related protein production and calcium transport in the lactating mammary gland, calcitonin secretion by the C-cells in the thyroid, maintenance of the hematopoietic stem cells (HSCs) niche in bone marrow and regulation of fluid secretion in the gastrointestinal tract.

The severe systemic abnormalities and perinatal lethality in CASR−/− mice limit the ability to discriminate between phenotypic abnormalities due to loss of CASR in a specific tissue from abnormalities secondary to these systemic alterations. The role of CASR in osteoblasts, for example, has been difficult to unequivocally establish. Earlier studies suggested that CASR may not be the relevant calcium receptor in osteoblasts, since the original CASR exon 5 deletion knockout mice fail to display any growth plate or bone phenotype when the concurrent hyperparathyroidism is corrected either by a “molecular parathyroidectomy” created by crossing glial cells missing 2-deficient mice onto the original “PTH rescued” CASR−/− mice which preserves translation and secretion of the extracellular domain which acts as a dominant negative factor to alter CASR function at distal sites leading to systemic alterations that indirectly impact upon osteoblast function. To test this possibility, we generated fragments of the extracellular domain of CASR and examined their ability to neutralize the effects of calcium-mediated activation of CASR in vitro and in vivo.

Materials and methods

Cloning and in vitro expression of the truncated mutants of the human CASR cDNA

To generate cDNAs of the extracellular domain of CASR, we performed PCR using full length human cDNA as a template and primer sets as follows. For the construction of V5-His-tagged truncated CASR vector, phCASR-V5-His, the hCASR exons 2–6 open reading frame was amplified by PCR using 5′-KpnI for ATG (5′-AGGTACCGAATGGCATTTATAGCTGCTGCTGGG) and 3′-XbaI 1392R (5′-AGTTAGTGCTCTCAGGACCTGCAC) and 3′-XbaI 1392R (5′-ATTC TAGATAGTGCTCTCAGGACCTGCAC). For the construction of phCASR-V5-His vector, we used the same forward primer with the reverse 3′-XbaI 1792R (5′-ATTC TAGATAGTGCTCTCAGGACCTGCAC). The PCR products were subcloned into high-level expression vector pDNA™3.1/V5-His (Invitrogen, Carlsbad, CA) and
the sequence of each cDNA was confirmed by direct DNA sequencing. We used pcDNA3.1-V5-His/lacZ as negative control for function analysis as well as a control for transfection, expression, purification, and detection.

Stable transfection of cells and protein analysis

We stably transfected the full-length receptor or truncated receptors, CASR\textsubscript{1-464} and CASR\textsubscript{1-598} cDNAs into HEK293 cells using modifications of previously described methods using TransFast Reagent (Promega, Madison, WI) and neomycin (Invitrogen).\textsuperscript{29} The truncated CASR proteins, or negative control LacZ were purified by ProBond Purification System (Invitrogen) according to the manufacturer’s protocol under native condition. The imidazole-eluted fractions were dialyzed against PBS buffer. Conditioned medium or total cell lysates were collected in a lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl\textsubscript{2}, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM AEBSF (4-(2-aminooethyl) benzensulfonyl fluoride), 5 mM levanisole, 1x Complete\textsuperscript{TM} protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) after 72 h of culture, and protein concentrations were determined by a DC Protein Analysis Kit (Bio-Rad, Hercules, CA). Western blot analysis were carried out as described previously (30) using 7% gels and an anti-V5 monoclonal antibody (mAb) (Invitrogen) or an anti-mouse CASR-specific mAb (ADD, provided by NPS Pharmaceuticals, Salt Lake City, UT) followed by appropriate secondary Abs for ECL detection (Amersham Biosciences, Piscataway, NJ). The mouse monoclonal antibody ADD was raised to the peptide corresponding to amino acids 214–236 encoded by exon 4 of the human CASR (ADDYYGRPGIEKFREEAAEERDI).\textsuperscript{30}

Cell culture and luciferase activity assay

HEK293 cells were co-transfected with pcDNA3.hCASR or pBK-CMV-PTHr (the expression plasmid for the PTH receptor)\textsuperscript{31} and SRE-luciferase reporter gene plasmid and pCMV-\beta-gal plasmid using TransFast transfection reagent (1:2 DNA/TransFast transfection reagent; Promega Corp., Madison, WI) in Opti-MEM I reduced serum medium (Invitrogen). Quiescence of transfected cells was achieved in subconfluent cultures by removing the media and washing with Hanks’ balanced salt solution (Invitrogen) to remove residual serum followed by incubation for an additional 24 hours in serum-free quiescent media. The quiescent cells were stimulated with the presence or absence of purified recombinant proteins of truncated CASR and in the presence or absence calcium or PTH (Sigma, St. Louis, MO) at indicated concentration. Luciferase activity was assessed after eight hours of stimulation. The luciferase activity in cell extracts was measured using the luciferase assay system (Promega) following the manufacturer’s protocol using a BG-luminometer (Gem Biomedical, Inc., Hamden, CT).

Assay for ERK1/2 mitogen-activated protein kinase

Agonist stimulation was performed in quiescent cells with calcium as indicated at five minutes. After agonist treatment at the specified concentrations and duration, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped into 250 µl of lysis buffer (25 mM HEPES pH 7.2, 5 mM MgCl\textsubscript{2}, 5 mM EDTA, 1% Triton X-100, 0.02 tablet/ml of protease inhibitor mixture). Equal amounts of lysates were subjected to 10% SDS-PAGE, and phospho-ERK1/2 levels were determined by immunoblotting using anti-phospho-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology, Beverly, MA). To confirm that variations in the amount of ERK did not contribute to stimulated ERK activity, we used an anti-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology) to measure ERK levels. Representative blots are shown, and the results were verified in at least three independent experiments.

In vivo mouse studies

Mice were maintained and used in accordance with recommendations as described (National Research Council. 1985; Guide for the Care and Use of Laboratory Animals DHHS Publication NIH 86-23, Institute on Laboratory Animal Resources, Rockville, MD) and following guidelines established by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

After fasting for 5–6 hours, each 2–3-month-old wild-type C57BL/6J mouse was administered a single intraperitoneal injection of ether 15 mg/kg body weight of recombinant proteins of truncated CASR, CASR\textsubscript{1-464} or negative control LacZ, respectively. The blood samples were collected 30 minutes after the administration of purified recombinant proteins using a retroorbital bleeding technique for measurement of serum PTH levels.\textsuperscript{32} Serum PTH and calcium were measured using the Mouse Intact PTH ELISA Kit (Immunotopics, San Clemente, CA) and Calcium Liquicolor (Stanbio Laboratory, Boerne, TX) as described previously.\textsuperscript{21}

Statistics

We evaluated differences between groups by one-way analysis of variance. All values are expressed as means ± standard
error of mean. All computations were performed using the StatGraphics statistical graphics system (STSC, Inc., Rockville, MD).

Results
Expression and secretion of hCASR extracellular domain fragments

We generated C-terminal deletion CASR constructs, one lacking exon 7 (CASR$_{1-598}$) that encodes the transmembrane and intracellular domains and the other (CASR$_{1-464}$) lacking exon 7 as well as 5 and 6$^{33}$ (Figure 1a). The extracellular calcium-binding sites and the sites mediating homodimerization are located at first 500 amino acids of CASR.$^{34,35}$ Total cell lysates of HEK293 cells stably transfected with each truncated receptors in vitro respectively produced single products with the predicted molecular mass of $\sim$65 kDa and $\sim$80 kDa, which were larger than predicted from the nucleotide sequence (ie, $\sim$55 or $\sim$70 kDa), consistent with in vitro glycosylation of the translated products (Figure 1b). In contrast, full length CASR produced a $\sim$250 kDa band representing dimerized product as well as bands of 140 and 120 kDa, representing differentially glycosylated products as previously reported.$^{30}$ To confirm secretion of the extracellular domain recombinant proteins, we compared the proteins in total cell lysates and conditioned media derived from cells expressing full-length and truncated CASR constructs. Using anti-CASR antiserum, the expected size immunoreactive bands corresponding to CASR$_{1-464}$ and CASR$_{1-598}$ were observed in conditioned medium from HEK293 cells expressing the truncated receptors, whereas these bands were absent from conditioned medium derived from HEK293 cells expressing full-length CASR (Figure 1c). Similar results were obtained using the V5 antibody to detect the secreted proteins (Data not shown). This result indicated that these truncated recombinant proteins CASR$_{1-464}$ and CASR$_{1-598}$ are secreted into conditioned medium, similar to the $\Delta$exon7-CASR construct reported by Chang and colleagues$^{35}$ (Figure 1c).

Functional analysis of the extracellular domain of hCASR in vitro

To elucidate the function of the secreted CASR$_{1-464}$ and CASR$_{1-598}$, we purified the recombinant proteins and examined their effect on calcium-stimulated CASR signal transduction by measuring SRE-luciferase activity in HEK293 cells co-expressing CASR and a SRE-luciferase reporter construct. We found that CASR$_{1-598}$ resulted in a dose-dependent inhibition of 5 mM extracellular calcium stimulated CASR activation. Concentrations of CASR$_{1-464}$ as low as 20 $\mu$g/ml, resulted in significant reductions in CASR activation by extracellular calcium, with an EC50 of approximately 75 $\mu$g/ml (Figure 2a).

Next, we examined the ability of increasing extracellular calcium concentrations to overcome the inhibitory effects of CASR$_{1-464}$ (100 $\mu$g/ml) (Figure 2b). Increments in extracellular calcium resulted in a dose-dependent increase in activation of CASR, reaching a maximum effect at 15 mM. Increase calcium concentrations up to 20 mM were not able to overcome the inhibitory effect of CASR$_{1-464}$, suggesting that the inhibitory effect may not be due to chelation/sequestration of the cationic ligand by the truncated CASR. CASR$_{1-464}$ and CASR$_{1-598}$ had similar effects to inhibit extracellular calcium activation of CASR (Figure 2d). In addition, CASR$_{1-464}$ also blocked CASR-mediated ERK activation by extracellular calcium (Figure 2c).

As an additional control, we examined the effects of 5 mM extracellular calcium to stimulate CASR-dependent SRE-luc activity in conditioned media containing 100 $\mu$g/ml of recombinant LacZ protein. Additions of recombinant LacZ protein had no effect on calcium-stimulated CASR

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**Figure 1** Expression of truncated mutant human CASRs. **A** Schematic diagram of human CASR cDNA structure. CASR$_{1-464}$ contains exons 2 to 4 and CASR$_{1-698}$ contains exons 2 to 6, both lacking exon 7 that encodes the 7-transmembrane domain (7TM) and C-terminal region.”*:’ denotes the binding site for the anti-CASR ADD antibody in exon 4. **B** and **C** Expression of recombinant CASR proteins in total cell lysates (**B**) and conditioned media (**C**) by Western blots analysis with ADD antibody. Total cells lysates (**B**) or conditioned media (**C**) were isolated from HEK293 cells stably transfected with either full-length wild-type CASR or mutant CASR$_{1-598}$ and CASR$_{1-464}$ cDNAs as described in Materials and methods.

**Notes:** The differentially glycosylated receptor full length CASR is denoted by (*), the truncated mutant receptor CASR$_{1-464}$ by the arrow “*” and the truncated mutant receptor CASR$_{1-598}$ by the block arrow “*” are present in both cell lysates and conditioned media.

**Abbreviations:**

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activity (Figures 2a, 2c and 2d). In addition, we found that the dominant negative effect of the CASR ED is specific for CASR, since CASR\textsubscript{1-464} failed to inhibit agonist-stimulated luciferase activity in HEK293 cells transfected with PTH receptor (Figure 2e).

**Effects of the extracellular domain of hCASR in vivo**

To define the role of the purified recombinant protein of truncated CASR in regulating PTH secretion in the parathyroid gland, we respectively administrated purified recombinant CASR ED or LacZ proteins to adult mice. We assessed serum PTH and calcium concentrations after one hour. The administration of purified recombinant protein of CASR\textsubscript{1-464} at doses of 15 and 50 mg/kg body weight resulted in significant elevations of serum PTH levels in wild-type C57B6/J mice (Figure 3a). In contrast, administration of 15 mg/kg body weight of purified protein of LacZ did not alter serum PTH levels (Figure 3a). The percentage increment of PTH after administration of recombinant CASR\textsubscript{1-464} was 2.4-fold (Figure 3). In addition, total serum total calcium levels were significantly decreased after the administration of 50 mg/kg body weight of purified recombinant protein of CASR\textsubscript{1-464} (Figure 3b). We failed to detect CASR\textsubscript{1-464} in serum one hour after injection by either Western blotting or immunoprecipitation (data not shown).
Discussion

Developing successful strategies to knockout G-protein coupled receptors is often challenging. Although deletion of the entire gene is desirable, this may not be feasible with large genes, such as CASR, where a single exon encodes the 7TM and C-terminal domains and the remaining upstream exons encode the extracellular domain. This has lead to two divergent strategies to create CASR knockout mice, namely deletion of exon 5 that encodes critical portions of the extracellular domain and “floxing” the exon 7 which encodes the 7TM and C-terminal domains. These two approaches have resulted in differences in the severity of the bone phenotypes in CASR-/- and conditional CASRΔexon7 mice. Several explanations may account for these differences. One possibility is that alternative splicing of exon 5 in the extracellular domain leads to continued expression of a functional, but hypomorphic receptor; whereas the deletion of exon 7 results in complete absence of functioning CASR and a more severe phenotype. Another possibility is that conditional ablation of the exon 7 encoding the 7TM and C-terminal domains results in the secretion of an extracellular domain that interferes with the function of the wild-type receptor at other sites.

In the current study we examined the potential for the extracellular domain of CASR to modulate CASR function in vitro and in vivo. We designed two cDNA constructs encoding the extracellular domains CASRΔ1-464 and CASRΔ1-598, but lacking the transmembrane and C-terminal domains. Overexpression of these constructs resulted in the secretion of these recombinant proteins (Figure 1). In addition, they dose-dependently inhibit the ability of extracellular calcium to stimulate CASR-mediated signaling in vitro (Figure 2). We also found that administration of the recombinant protein of truncated CASR increased serum PTH level in vivo (Figure 3). A similar construct, Δexon7-CASR, was reported by Chang and colleagues to have no function as assessed by the inability of this construct to signal in response to extracellular cations in HEK293 cells co-transfected with full length CASR; but unlike our studies they did not directly evaluate the ability of the product of Δexon7-CASR to inhibit the function of CASR.

There are several possible mechanisms whereby a recombinant protein encoding the extracellular domain of CASR might inhibit CASR-mediated signaling. One possibility is that the calcium binding sites in the extracellular domain may sequester calcium and limit binding to CASR, thereby functioning as a true decoy receptor analogous to osteoprotegerin (OPG) binding to receptor activator of NF-κB ligand (RANKL) and inhibiting RANK activation. The inability of increasing concentrations of calcium to overcome the inhibitory effects of truncated CASR argues against this mechanism. Alternatively, CASRΔ1-464 may act as a dominant negative modulator that somehow disrupts calcium-dependent activation of wild-type CASR. There are many examples of naturally occurring decoy receptors that interfere with the function of a variety of pathways, including OPG, a secreted decoy receptor that binds to RANKL and secreting frizzled-related proteins (sFRPs) which are truncated forms of frizzled receptor that act as decoy
receptors for Wnt signaling. In addition, analogous to our studies, a purified recombinant GPR56 extracellular domain protein has been shown to inhibit glioma cell adhesion,60 a peptide derived from the sixth transmembrane domain of the adrenergic receptor inhibits receptor activation and dimerization,61 and a soluble chimeric fragment of the E-prostanoid receptors 2 (EP2) is reported to antagonize PGE2-mediated EP signaling in prostate cells in vitro.62 Further investigations will be needed to determine the mechanism whereby the extracellular domain of CASR interferes with CASR function.

We did not directly examine the alternative interpretation that a splice variant of CASR lacking exon 5 functions as a hypomorphic mutation. An alternatively spliced CASR is reported to exist, but its function has not been clearly demonstrated. For example, Oda and colleagues45 have recently described an alternatively spliced form of the CASR in keratinocytes that lacks exon 5, producing an in-frame deletion of 77 amino acids, similar to the alternately splicing transcripts in global CASR knockout mice. The truncated CASR, however, was inactive when transfected into HEK293 cells or keratinocytes, but it interfered with the function of a coexpressed full-length CASR.41 In addition, human genetic studies do not support the idea of hypomorphic mutations, since mutations leading to the complete functional deletion of CASR do not have a more severe phenotype than mutations leading to expression of an inactive receptor and parathyroidectomy cures the skeletal abnormalities in humans with neonatal severe hyperparathyroidism (HSHPT).44 Indeed, a patient with a mutation resulting in a truncated CASR lacking the 7TM and C-terminal domains had normal bone morphology and mineralization after parathyroidectomy,45 consistent with the observation in exon 5 CASRΔexon7 mice that demineralization and other skeletal abnormalities are due to the effects of hyperparathyroidism and not to CASR deficiency per se.21,22

Given that both existing CASR mutant mouse models have limitations, additional studies are needed to fully understand the role of CASR in bone. In particular, additional proof is needed that the alternatively spliced exon 5 deleted CASR mutant functions as a hypomorphic mutation in bone, which might be evaluated by the overexpression of a mutant cDNA to attempt rescue the bone phenotype in conditional CASRΔexon7 mice;23 or alternatively by siRNA-mediated silencing of this isoform in CASRΔexon7 mice, which should result in a more severe bone phenotype. On the other hand, since the floxed exon 7 CASR mice produce the extracellular domain of CASR encoded by exons 2–612,25 that can disrupt the function of CASR, the tissue specificity in conditional CASR knockout mice may be compromised by the effect of the dominant negative CASR extracellular domain could to interfere with CASR function in other tissues that express CASR (such as the parathyroid gland or bone marrow derived cells), and in doing so, cause either systemic or paracrine alterations that indirectly impact upon tissues or cell types that do not express CASR.

If so, the purported osteoblastic abnormalities in the “conditional” CASR null mice might not arise from loss of CASR in osteoblasts per se, but may be due to an indirect affect on osteoblastic function of systemic or paracrine abnormalities arising from the production of a secreted extracellular domain of CASR from other tissues and cell types targeted by “leaky promoters”, and resulting in the release of factors (such as PTH or PTHrp) that indirectly impact on osteoblast function.60 Although the original publication by Chang and colleagues25 did not report serum biochemical measurements in mice with “bone-specific deletion” of CASRΔexon7 using 2.3 Col (1)-Cre and osterix-Cre, subsequent reports indicate that these mice appear to have PTH-dependent hypercalcemia (11.5–13 mg/dL).27 Since neither promoters limit expression to osteoblasts,47–49 these systemic effects could arise from deletion of CASR in nonosteoblastic sites. Indeed, use of the more osteoblast-specific osteocalcin promoter to ablate CASRΔexon7 in mice resulted in no reported bone phenotype.50 While we do not know if the levels of the extracellular domain of CASR necessary to disrupt CASR function is achieved in conditional floxed CASRΔexon7 mice, it will be important in future studies to measure and assess the potential effects of the circulating extracellular domain of CASR in conditional knockout mice.

In conclusion, we show that the extracellular domain of CASR can inhibit extracellular calcium-induced cell signaling through CASR in vitro and in vivo. Moreover, this dominant negative action of the extracellular domain of CASR provides an alternative mechanism to explain the more extensive phenotype observed in mouse models that have selectively deleted the transmembrane domain and C-terminus of CASR in the skeleton. Distinguishing this from the other hypothesized mechanism, namely that the global CASR null mice lacking exon 5 are hypomorphic,25,27 will require additional approaches, such as knockout of the entire CASR gene, silencing the alternatively spliced mutant CASR receptor in CASRΔexon7 mice, overexpressing the exon 5 alternatively spliced CASR isoform in bone of conditional CASRΔexon7 mice, assessing circulating levels of the extracellular domain of CASR in conditional CASRΔexon7 mice, and/or
transgenic overexpression of the extracellular domain of CASR to extend the findings of the current investigations. Regardless, given the inconsistencies regarding CASR function in osteoblasts with existing models, further studies are needed to fully understand the function of CASR in bone.

Disclosures
This work was supported by NIH R01-AR37308 (LDQ). The authors report no conflicts of interest in this work. MP and LDQ conceived and designed the experiments. MP, JC, and WZ performed the experiments. MP and LDQ analyzed the data and wrote the paper.

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