Osteoprotegerin Ligand Induces β-Casein Gene Expression through the Transcription Factor CCAAT/Enhancer-binding Protein β*

Osteoprotegerin ligand (OPGL, also known as RANKL), a member of the tumor necrosis factor superfamily, is essential for mammary gland development during pregnancy in addition to key roles in the immune system and bone development. Here we show that OPGL induces β-casein transcription through the CCAAT/enhancer-binding protein β (C/EBPβ). In both HC11 cell lines and primary mammary epithelial cells, OPGL stimulation triggers rapid nuclear translocation of C/EBPβ, which is critical for the expression of the β-casein gene. Mutation of C/EBPβ binding sites in the β-casein gene promoter completely abrogated OPGL-induced β-casein promoter activity. By contrast, OPGL stimulation did not result in STAT5 phosphorylation. In vivo immunohistochemistry studies further demonstrated defective nuclear translocation of C/EBPβ, but normal STAT5 activation, in OPGL-deficient mice. These data show that OPGL is a critical activator of β-casein gene expression via the transcription factor C/EBPβ. Our data provide new insights into the understanding of the molecular events involved in milk protein gene expression.

Osteoprotegerin ligand (OPGL)\(^1\); also called ODF, RANKL, and TRANCE) is a tumor necrosis factor-related cytokine that regulates osteoclast differentiation from hematopoietic precursors. Moreover, OPGL is required for the activation of mature osteoclasts (1–4). OPGL-deficient mice exhibit severe osteopetrosis and failure in tooth eruption due to a complete absence of osteoclasts and defective bone remodeling (5). OPGL also regulates the function and survival of dendritic cells. These functions of OPGL are mediated via binding to its receptor RANK (receptor activator of NF-κB) (6). In addition, it has been shown that OPGL and its receptor RANK are expressed in mammary epithelial cells, and both are required for survival and proliferation of mammary epithelial cells during pregnancy (7). Mice lacking OPGL or RANK display a complete defect in lobuloalveolar development during pregnancy and a block in expression of milk proteins such as β-casein, resulting in the death of offspring. These findings indicate that OPGL and RANK are critical for the development of a lactating mammary gland during pregnancy. Whether the defect in lactogenesis is a direct consequence of the OPGL/RANK deficiencies or secondary to the observed developmental block is not known.

Similar to OPGL and RANK, prolactin can induce lobuloalveolar development in the mammary gland and stimulate postpartum lactogenesis. Binding of prolactin to its receptor results in the phosphorylation and activation of the Janus tyrosine kinase JAK2 and signal transducers and activators of transcription (STAT) 5. Phosphorylated/activated STAT5 translocates to the nucleus where it binds to γ-interferon activation sites (9) and induces the transcription of target genes such as β-casein. Moreover, stimulation of HC11 cells with lactogenic hormones enhances the expression of β-casein gene in vitro (8). However, STAT5a-deficient mice showed normal β-casein gene expression (10), suggesting that β-casein expression is regulated by other transcription factor(s).

The promoter region of the β-casein gene contains four binding sites for C/EBPβ, implicating C/EBPβ as an important regulator of β-casein gene expression in mammary epithelial cells (11). C/EBPs are a highly conserved family of DNA-binding proteins involved in the regulation of target genes for growth control and differentiation in multiple cell types (12–14). The C/EBP isoforms α, β, and δ are expressed in the mammary gland and developmentally regulated during pregnancy, lactation, and involution (15). C/EBPα mRNA content is relatively low throughout pregnancy and lactation. Mammary C/EBPβ mRNA levels are elevated during pregnancy, decline slightly during lactation, and increase again at the onset of involution. C/EBPβ mRNA levels are markedly induced in cultured epithelial cells during growth arrest (16). Importantly, C/EBPβ-null mice exhibit defective lobuloalveolar proliferation and milk protein gene expression (17, 18). However, the upstream signals that control milk protein expression via C/EBPβ are not known.

In this study, we investigated the role of OPGL in mammary lactogenesis using primary and HC11 cells. We show that OPGL stimulation directly enhances β-casein gene expression. OPGL induces the translocation of C/EBPβ, but not STAT5, from the cytoplasm to the nucleus, resulting in the expression of β-casein gene. These results identify OPGL as a novel factor required for the expression of β-casein gene in mammary gland epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HC11 cells were obtained from Dr. Chul-Sang Lee (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). Cells were maintained in RPMI 1640 medium containing 10

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\1 The abbreviations used are: OPGL, osteoprotegerin ligand; PRL, prolactin; C/EBP, CCAAT/enhancer-binding protein; RANK, receptor activator of NF-κB; JAK, Janus tyrosine kinase; STAT, signal transducers and activators of transcription; RT, reverse transcription; WT, luciferase construct containing the sequences from −331 to −1 of the wild-type rat β-casein promoter; MT, respective mutant construct containing several mutated nucleotides in the β-casein gene promoter.
Complete Protease Inhibitor Mixture tablet (Roche Molecular Biochemicals) was washed with cytoplasmic extraction buffer without Nonidet P-40. Nucleophosmin (specific for Tyr-694, New England Biolabs) and C/EBP binding sites were incubated with polyclonal antibodies to phospho-STAT5 (obtained as follows. Cells were lysed in extraction buffer (10 mM Tris HCl, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM Na3VO4, and 0.1% Triton X-100) filtered through a 40-μm nylon mesh filter, and allowed to settle for 1 min. The supernatant containing nonadherent cells were removed. The adherent cells were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 0.5% dexamethasone, and incubated at 37 °C for 1 h. After washing and centrifugation at 1000 rpm for 1 h, cells were incubated at 37 °C for 4 h in buffer A containing 1 μg/ml collagenase. Following digestion, cells were centrifuged, redigested in buffer A containing 2 μg/ml collagenase, resuspended in buffer A filtered through a 40-μm nylon mesh filter, and allowed to settle for 1 min. Cell pellets were resuspended in buffer A before centrifugation and nuclear translocation experiments, cells were placed in serum-free medium for 24 or 48 h prior to OPGL or prolactin stimulation. For RT-PCR experiments, cells were grown in RPMI 1640 medium containing 5% charcoal-stripped fetal bovine serum for 18 h prior to each treatment.

Fluorescence-activated Cell Sorting Analysis—HC11 cells or primary mammary epithelial cells from 8-week-old virgin mice were stained with OPGL-fluorescein isothiocyanate and analyzed by flow cytometry to detect RANK expression. RT-PCR—HC11 or primary cells were stimulated with either OPGL or prolactin for 24 h. Total RNA was isolated using TRIzol according to the instructions of the manufacturer. RNA (1 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and random hexamers (Amersham Biosciences, Inc.). The PCR amplification was carried out using the following primers: for β-casein, sense (5′-AATCCAGACTCATCGTACAAG-3′) and antisense (5′-GGTTGTTCCAGTGGCTATAAC-3′); for β-actin, sense (5′-CAGTGGCCCATGCTTCCGTCAAG-3′) and antisense (5′-GCTGTCGCCTTCACCGTTCT-3′). Total RNA from 14.5-day pregnant mouse mammary tissue was used as a positive control.

Nuclear and Whole Cell Protein Extraction—Nuclear extracts were prepared as described previously (19). Briefly, cells were harvested, washed with phosphate-buffered saline, and lysed on ice for 15 min in cytoplasmic extraction buffer (10 mM HEPES (pH 7.6), 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.5% dithiothreitol, 1 mM Na3VO4, and Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)). Cells were centrifuged at 12,000 × g at 4 °C for 1 min and washed with cytoplasmic extraction buffer without Nonidet P-40. Nuclear pellets were resuspended in nuclear extraction buffer (20 mM Tris (pH 8.0), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5% dithiothreitol, 1 mM Na3VO4, and Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)), incubated on ice for 30 min with occasional vortexing, and then centrifuged at 12,000 × g at 4 °C for 5 min. Whole cell extracts were obtained as follows. Cells were lysed in extraction buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, and Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)), incubated at 4 °C for 30 min by shaking, and then cleared by centrifugation. Protein concentrations were determined using a bicinchoninic acid assay.

Immunoblotting—Equal amounts of whole cell extracts or nuclear extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies reactive to STAT5α (Upstate Biotechnology, Lake Placid, NY), phosho-STAT5 (specific for Tyr-694; New England Biolabs, Beverly, MA), or C/EBPβ (C-19; Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences, Inc.).

**RESULTS**

**OPGL Directly Induces β-Casein Gene Expression in Mammary Gland Epithelial Cells**—We have previously shown that opgl−/− mice exhibit a complete block in lobuloalveolar mammary gland development and an absence of β-casein gene expression during pregnancy. Local injection of recombinant OPGL rescued both defects (7). It was not clear whether impaired expression of β-casein gene in opgl−/− mice was secondary to the developmental defect in lobuloalveolar differentiation or an intrinsic defect in β-casein gene expression. To address whether OPGL is capable of directly inducing the expression of β-casein gene on mammary epithelial cells, we analyzed the effect of OPGL in HC11 mammary gland cell lines and primary mouse mammary epithelial cells from virgin C57BL/6 mice. Both, HC11 and primary cells express the OPGL receptor RANK on the cell surface (Fig. 1, A and C).

To investigate the transcriptional induction of the milk protein gene β-casein by OPGL, HC11 and primary mammary epithelial cells were treated with either OPGL or the lactogenic hormone prolactin, which has been previously shown to induce β-casein gene expression in mammary gland epithelial cells (8). Following stimulation for 24 h, β-casein gene expression was analyzed by RT-PCR. Intriguingly, expression of β-casein mRNA was increased in response to OPGL in HC11 cells (Fig. 1B). Importantly, β-casein mRNA expression was also significantly increased in primary mammary epithelial cells following OPGL and prolactin stimulation. Thus, similar to the established lactogenic hormone prolactin, OPGL can directly trigger β-casein gene expression in mammary epithelial cells.

**STAT5 Is Independent of the β-Casein Gene Expression by OPGL**—Prolactin stimulation results in JAK2 activation and phosphorylation of STAT5. Phosphorylated STAT5 then translocates to the nucleus and binds to γ-interferon activation sites present in the promoter regions of milk protein genes (20). Since OPGL stimulation induced β-casein gene expression, we analyzed the signaling intermediates that mediate the expression of milk protein genes by OPGL.

To test whether STAT5 acts downstream of OPGL-RANK signaling for the expression of β-casein, we examined phosphorylation/activation of STAT5 following OPGL treatment in HC11 cells. As shown in Fig. 2A, STAT5 was rapidly phosphorylated 5 min after prolactin treatment, and STAT5 phosphorylation was sustained for 1 h. In contrast, OPGL stimulation did not result in any detectable phosphorylation of STAT5, even 1 h following OPGL stimulation. Similar results were also obtained in studies using human T47D breast cancer cells (Fig. 2B), which also express RANK (21). STAT5 protein levels did not change over a 72-h treatment period with OPGL (Fig. 2C).

These results show that OPGL-RANK do not utilize the JAK-STAT5 pathway for the induction of β-casein. Since β-casein gene expression was completely abolished in opgl−/− mice (7) and OPGL-RANK signaling is independent of STAT5, we speculated that OPGL could induce β-casein gene expression through C/EBPβ.

**OPGL Triggers Nuclear Translocation of the CCAAT/Enhancer-binding Protein β**—Mutational analysis of the β-casein gene promoter suggested that C/EBPβ sites are essential for hormone-induced β-casein gene expression (11). Moreover, it has been reported that C/EBPβ is critical for the expression of β-casein gene during C/EBPβ gene deletion studies (17, 18). Since β-casein gene expression was completely abolished in opgl−/− mice (7) and OPGL-RANK signaling is independent of STAT5 (Fig. 2, A and B), we speculated that OPGL could induce β-casein gene expression through C/EBPβ. Thus, we first investigated whether OPGL treatment of HC11 cells and primary mammary epithelial cells would result in the nuclear translocation of C/EBPβ.
Fig. 1. OPGL induces the expression of β-casein mRNA in HC11 (A and B) and primary mammary epithelial cells (C and D). Fluorescence-activated cell sorting analysis of HC11 (A) and primary mammary epithelial (C) cells with OPGL-fluorescein isothiocyanate (OPGL-FITC) shows RANK expression (solid line). The dotted lines represent background fluorescence. HC11 and primary cells were treated for 24 h without (−) or with (+) OPGL (1 μg/ml) or prolactin (PRL, 5 μg/ml). β-Casein mRNA expression was detected using RT-PCR (B and D). Total RNA from pregnant mouse mammary tissue was used as a positive control (d14.5). β-Actin is shown as a mRNA loading control. One result representative of three experiments is shown.

Fig. 2. OPGL does not affect the activation/phosphorylation and expression of STAT5. A and B, STAT5 is not phosphorylated following OPGL stimulation. Serum-starved HC11 (A) and T47D (B) cells were treated with prolactin (PRL, 5 μg/ml) and OPGL (1 μg/ml) for the indicated time periods. Phospho-STAT5 (P-Stat5) (upper panel) and total STAT5α (lower panel) were detected by Western blotting. C, expression of total STAT5 protein in HC11 cells following stimulation with OPGL (1 μg/ml). Western blot analysis was performed with an anti-STAT5α antibody. One result representative of three experiments is shown.

Fig. 3. OPGL induces nuclear translocation of C/EBPβ, but not STAT5, in HC11 cells. HC11 cells were treated with either OPGL (1 μg/ml) or prolactin (PRL, 5 μg/ml) for the indicated times, and nuclear extracts were prepared as described under “Experimental Procedures.” Nuclear proteins were analyzed by Western blotting for the presence of STAT5 and C/EBPβ (A) and quantitated by densitometry scanning for C/EBPβ levels (B). Relative protein levels are shown as mean values ± S.E. of three separate experiments.

cation of C/EBPβ. Indeed, OPGL stimulation induced the nuclear translocation of C/EBPβ. In HC11 cells, nuclear translocation was first observed around 30 min following OPGL stimulation, and nuclear translocation of C/EBPβ was sustained for 3 h (Fig. 3). Similar results were observed in primary mammary epithelial cells from 8-week-old virgin mice (Fig. 4), indicating that OPGL is a physiological activator of C/EBPβ-mediated β-casein gene expression. OPGL stimulation did not result in STAT5 translocation into the nucleus, a finding that is consistent with our data that STAT5 is not phosphorylated in response to OPGL stimulation. In contrast, prolactin treatment induced the nuclear translocation of both C/EBPβ and STAT5 in HC11 and primary epithelial cells (Figs. 3 and 4). These results show that OPGL stimulation results in the specific activation and prolonged nuclear accumulation of the transcription factor C/EBPβ.

C/EBPβ Binding Sites in the β-Casein Promoter Are Crucial for OPGL-regulated β-Casein Promoter Activity—To address whether OPGL-induced β-casein gene expression is mediated by the C/EBPβ transcription factor, HC11 cells were trans-
abolishes C/EBP/H9252 cells were isolated from virgin mice and treated with either OPGL (1 μg/ml) or prolactin (PRL, 5 μg/ml) for the indicated times. Expression levels of C/EBPβ and STAT5 were analyzed by immunoblotting (A) and quantitated by densitometry scanning for nuclear C/EBPβ levels of C/EBP/H9252 and quantitated nucleotides in the C/EBP/H9252 showed to abolish binding sites of the C/EBP/H11002 infected with either a luciferase construct containing the se-

Fig. 4. Nuclear translocation of C/EBPβ, but not STAT5, in primary mammary epithelial cells. Primary mammary epithelial cells were isolated from virgin mice and treated with either OPGL (1 μg/ml) or prolactin (PRL, 5 μg/ml) for the indicated times. Expression levels of C/EBPβ and STAT5 were analyzed by immunoblotting (A) and quantitated by densitometry scanning for nuclear C/EBPβ (B). Mean values ± S.E. of three separated experiments are shown.

Dexamethasone (Dex) significantly induced β-casein gene promoter activity in WT-transfected HC11 cells to levels that were comparable to that of prolactin plus dexamethasone stimulation (Fig. 5). In contrast, mutation in the C/EBPβ binding sites of the β-casein gene promoter completely abolished OPGL-induced promoter activity in HC11 cells (Fig. 5). These results show that C/EBPβ binding sites in the β-casein promoter region are critical for OPGL-induced β-casein gene expression and that OPGL directly induces β-casein gene expression through the C/EBPβ transcription factor.

Impaired Nuclear Translocation of C/EBPβ in opgl−/− Mice—Since our in vitro data in cell lines and primary epithelial cells showed that OPGL induces C/EBPβ translocation, we analyzed the status of C/EBPβ in the mammary glands of pregnant opgl−/− mice in vivo. As shown in Fig. 6A, C/EBPβ is localized in the nucleus of wild-type epithelial cells at 1 day of lactation. However, C/EBPβ was not detectable in the nucleus of mammary gland epithelial cells of opgl−/− females at 1 day of lactation (Fig. 6B). In contrast to defective nuclear translocation of C/EBPβ in opgl−/− females, the levels and subcellular distributions of phospho-STAT5 (Fig. 6, C and D) were comparable between opgl−/− and wild-type mammary gland epithelial cells. These data show that OPGL has no apparent role in the phospho-STAT5 signaling pathway, but OPGL is a specific activator of nuclear accumulation and translocation of C/EBPβ in vivo.

DISCUSSION

OPGL is a key regulator of skeletal calcium release and the development of a lactating mammary gland. In addition, opgl−/− mice exhibit a complete absence of β-casein gene expression during pregnancy (7). It was not clear whether the impaired expression of β-casein gene in opgl−/− mice resulted from either underdevelopment of the mammary gland or was based on an intrinsic defect in β-casein gene expression. Here we demonstrate that OPGL directly induces the expression of the β-casein gene. This effect of OPGL is mediated by the transcription factor C/EBPβ.

Our data show that both prolactin and OPGL are required for appropriate β-casein gene expression (7, 20). Several nu-

Fig. 5. Absolute requirement of C/EBPβ binding sites for OPGL-induced β-casein gene promoter activity. Wild-type rat β-casein gene promoter luciferase constructs (−331 to −1) (white bars) or a similar construct with a mutation in C/EBPβ binding sites (black bars) were transiently transfected into HC11 cells. Transfected HC11 cells were then treated for 48 h with 1 μg/ml OPGL, 1 μg/ml OPGL plus 0.1 μM dexamethasone (Dex), or 5 μg/ml prolactin (PRL) plus 0.1 μM dexamethasone. Luciferase reporter activity was normalized to Renilla luciferase activity. Results are shown as mean values ± S.E. of three separate transfection experiments.

We stimulated WT- or MT-transfected HC11 cells with either OPGL alone or OPGL plus dexamethasone. OPGL stimulation fected with either a luciferase construct containing the sequences from −331 to −1 of the wild-type rat β-casein promoter (WT) or a respective mutant construct containing several mutated nucleotides in the β-casein gene promoter (MT) that abolishes C/EBPβ binding. These mutations in the C/EBPβ binding sites of the β-casein gene promoter have also been shown to abolish β-casein gene expression in response to lactogenic hormones (11).

We stimulated WT- or MT-transfected HC11 cells with either OPGL alone or OPGL plus dexamethasone. OPGL stimulation significantly induced β-casein gene promoter activity in WT-transfected HC11 cells to levels that were comparable to that of prolactin plus dexamethasone stimulation (Fig. 5). In contrast, mutation in the C/EBPβ binding sites of the β-casein gene promoter completely abolished OPGL-induced promoter activity in HC11 cells (Fig. 5). These results show that C/EBPβ binding sites in the β-casein promoter region are critical for OPGL-induced β-casein gene expression and that OPGL directly induces β-casein gene expression through the C/EBPβ transcription factor.

Impaired Nuclear Translocation of C/EBPβ in opgl−/− Mice—Since our in vitro data in cell lines and primary epithelial cells showed that OPGL induces C/EBPβ translocation, we analyzed the status of C/EBPβ in the mammary glands of pregnant opgl−/− mice in vivo. As shown in Fig. 6A, C/EBPβ is localized in the nucleus of wild-type epithelial cells at 1 day of lactation. However, C/EBPβ was not detectable in the nucleus of mammary gland epithelial cells of opgl−/− females at 1 day of lactation (Fig. 6B). In contrast to defective nuclear translocation of C/EBPβ in opgl−/− females, the levels and subcellular distributions of phospho-STAT5 (Fig. 6, C and D) were comparable between opgl−/− and wild-type mammary gland epithelial cells. These data show that OPGL has no apparent role in the phospho-STAT5 signaling pathway, but OPGL is a specific activator of nuclear accumulation and translocation of C/EBPβ in vivo.

DISCUSSION

OPGL is a key regulator of skeletal calcium release and the development of a lactating mammary gland. In addition, opgl−/− mice exhibit a complete absence of β-casein gene expression during pregnancy (7). It was not clear whether the impaired expression of β-casein gene in opgl−/− mice resulted from either underdevelopment of the mammary gland or was based on an intrinsic defect in β-casein gene expression. Here we demonstrate that OPGL directly induces the expression of the β-casein gene. This effect of OPGL is mediated by the transcription factor C/EBPβ.

Our data show that both prolactin and OPGL are required for appropriate β-casein gene expression (7, 20). Several nu-
clear DNA binding factors that bind specifically to the \( \beta \)-casein promoter have been identified (11, 22). Among these, the best characterized transcription factor is STAT5 (23), and the JAK-STAT pathway is essential for prolactin-induced expression of the milk protein genes \( \beta \)-casein and whey acidic protein (20). However, in contrast to prolactin, OPGL stimulation does not induce STAT5 phosphorylation or translocation of STAT5 to the nucleus in HC11 and primary mammary epithelial cells. Moreover, phospho-STAT5 was detectable at normal levels in OPGL-deficient mammary epithelial cells in vivo. Thus, OPGL-RANK do not activate the JAK2-STAT5 pathway.

Another transcription factor required for the expression of the \( \beta \)-casein gene is C/EBP\( \beta \) (24). The \( \beta \)-casein gene promoter contains four C/EBP\( \beta \) binding sites that are important for promoter activity in response to prolactin (11, 24). Furthermore, studies using C/EBP\( \beta \)-deficient mice revealed that C/EBP\( \beta \) is critical for the expression of milk protein genes such as \( \beta \)-casein and for mammary gland development in vivo (17, 18, 25). Although it is clear that C/EBP\( \beta \) plays an essential role in the transcription of the \( \beta \)-casein gene, no upstream activator has been identified. Our study shows that OPGL treatment results in nuclear translocation of C/EBP\( \beta \) in both HC11 and primary mammary epithelial cells (Figs. 3 and 4). Moreover, mutational analyses of C/EBP\( \beta \) binding sites in the \( \beta \)-casein gene promoter confirmed that C/EBP\( \beta \) binding sites are crucial for the OPGL-induced \( \beta \)-casein gene promoter activity. However, OPGL is not the sole activator of C/EBP\( \beta \) since prolactin also induced nuclear translocation of C/EBP\( \beta \) (Figs. 3 and 4). It will be interesting to test whether prolactin acts upstream of OPGL in vivo and whether prolactin-induced binding of C/EBP\( \beta \) to the \( \beta \)-casein promoter depends on OPGL expression.

Two important transcriptional factors, C/EBP\( \beta \) and STAT5, have been identified that control \( \beta \)-casein expression. Whereas gene deletion studies revealed an essential role of C/EBP\( \beta \) for \( \beta \)-casein gene expression, \( \beta \)-casein gene expression is relatively normal in STAT5\( \alpha \)-deficient mice. In our present study, both OPGL and prolactin can induce nuclear translocation of C/EBP\( \beta \). It is unlikely that opgl\(^{-/-}\) mice have a defect in prolactin activity because opgl\(^{-/-}\) mice are fertile in contrast to prolactin receptor-deficient mice that are sterile (26). Moreover, loss of OPGL expression does not alter nuclear translocation of phospho-STAT5 in mammary glands of pregnant females (Fig. 5D). However, we cannot exclude the possibility that another transcription factor regulated by OPGL might cooperate with C/EBP\( \beta \) in the regulation of \( \beta \)-casein gene expression (Fig. 7).

In summary, we demonstrate for the first time that OPGL, the mediator of skeletal calcium release, directly induces the expression of \( \beta \)-casein gene. Mechanistically, OPGL stimulation of mammary epithelial cells results in the nuclear translocation of the transcription factor C/EBP\( \beta \). Thus, C/EBP\( \beta \) is a downstream target of OPGL required for the expression of \( \beta \)-casein milk protein gene in mammary gland epithelial cells.

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REFERENCES

1. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Treee, M. C., DuRose, R. F., Cosman, D., and Galibert, L. (1997) Nature 390, 175–179
2. Lacey, D. L., Timms, E., Tan, H.-L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliot, R., Colombino, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eti, A., Qian, Y.-X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., and Delaney, G. J. (1998) Cell 93, 165–176
3. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Volkodavskiya, M., Hanafusa, H., and Choi, Y. (1997) Mol. Cell 4, 1014–1049
4. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., and Murakami, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3597–3602
5. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H.-L., Timms, E., Capparelli, C., Mori, S., Oliveira-dos-Santos, A. J., Van, G., Hile, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mark, T. W., Boyle, W. J., and Penninger, J. M. (1999) Nature 397, 315–321
6. Wong, R. B., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) J. Exp. Med. 186, 2075–2080
7. Fata, J. E., Kong, Y. Y., Li, J., Sasaki, T., Irie-Sasaki, J., Moorehead, B. A., Elliot, R., Scully, S., Vours, E. B., Lacey, D. L., Boyle, W. J., Khoo, R., and Penninger, J. M. (2000) Cell 103, 41–50
8. Ball, R. K., Friis, R. R., Schonenberger, C. A., Doppler, W., and Groner, B. (1988) EMBO J. 7, 2089–2095
9. Ibe, J. N. (1996) Cell 84, 331–334
10. Liu, X., Robinson, G. W., Wagner, K.-U., Garrett, L., and Wynshaw-Boris, A. (1997) Genes Dev. 11, 179–186
11. Doppler, W., Welte, T., and Philipp, S. (1995) J. Biol. Chem. 270, 17062–17069
12. Bikennmier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschutz, W. H., and McKnight, S. L. (1989) Genes Dev. 3, 1146–1156
13. Cao, Z., Unke, R. M., and McKnight, S. L. (1991) Genes Dev. 5, 1538–1552
14. Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1998) Genes Dev. 12, 168–181
15. Giglott, A. P., and DeWille, J. W. (1998) J. Cell. Physiol. 174, 232–239
16. O’Rourke, J., Yuan, R., and DeWille, J. (1997) J. Biol. Chem. 272, 6291–6296

Fig. 7. Schematic representation of OPGL signaling for \( \beta \)-casein expression in mammary gland epithelial cells. OPGL binding to RANK leads to translocation of C/EBP\( \beta \) from the cytoplasm to the nucleus and expression of C/EBP\( \beta \) target genes such as \( \beta \)-casein. Prolactin binding also triggers nuclear translocation of C/EBP\( \beta \) as well as activation of the JAK2-STAT5 signaling pathway. Hypothetically, another OPGL-RANK-induced signaling pathway (dotted line) may lead to the induction of nuclear translocation of a second putative transcription factor (TF) that cooperates with C/EBP\( \beta \) to control expression of \( \beta \)-casein gene.
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17. Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. (1998) Genes Dev. 12, 1907–1916
18. Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J., and Rosen, J. M. (1998) Genes Dev. 12, 1917–1928
19. Geymayer, S., and Doppler, W. (2000) FASEB J. 14, 1160
20. Hennighausen, L., Robinson, G. W., Wagner, K.-U., and Liu, X. (1997) J. Biol. Chem. 272, 7567–7569
21. Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T. (1999) Endocrinology 140, 4451–4458
22. Schmitt-Ney, M., Doppler, W., Ball, R. K., and Groner, B. (1991) Mol. Cell. Biol. 11, 3745–3755
23. Wakao, H., Gouilleux, F., and Groner, B. (1994) EMBO J. 13, 2182–2191
24. Raught, B., Liao, W. S., and Rosen, J. M. (1995) Mol. Endocrinol. 9, 1223–1232
25. Seagroves, T. N., Lydon, J. P., Hovey, R. C., Vonderharr, B. K., and Rosen, J. M. (2000) Mol. Endocrinol. 14, 359–368
26. Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., and Kelly, P. A. (1997) Genes Dev. 11, 167–178