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Integration of the NfκB p65 Subunit Into the Vitamin D Receptor Transcriptional Complex: Identification of p65 Domains That Inhibit 1,25-Dihydroxyvitamin D3-Stimulated Transcription

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Abstract Resistance to the action of vitamin D (D) occurs in response to tumor necrosis factor-α (TNF-α), an effect mediated by nuclear factor kappa B (NfκB). To determine the mechanism of NfκB inhibition of D-stimulated transcription, chromatin immunoprecipitation assays (CHIP) were done in osteoblastic ROS 17/2.8 cells that had been treated with TNF-α or transfected with the p65 subunit of NfκB. These treatments caused stable incorporation of p65 into the transcription complex bound to the vitamin D response element (VDRE) of the osteocalcin promoter. Deletion analysis of p65 functional domains revealed that the p65 N-terminus and a midmolecular region were both required for the inhibitory action of p65. Pull-down assays were done using an immobilized glutathione S-transferase (GST)-VDR fusion protein to study the effect of p65 on VDR binding to steroid coactivator-1 (SRC-1), a major D-dependent coactivator. p65 inhibited VDR-SRC-1 binding in a dose-dependent manner. Mutations of p65 that abrogated the inhibitory effect on D-stimulated transcription also failed to inhibit VDR-SRC-1 binding. The inhibitory effect of p65 on VDR transactivation was not due to recruitment of a histone deacetylase (HDAC), since inhibition was not relieved by the HDAC inhibitors sodium butyrate or trichostatin A. Overexpression of SRC-1 or the general coactivators, Creb binding protein or SRC-3, also failed to relieve p65 inhibition of transcription. In addition, Chip assays revealed that TNF-α treatment prevented D recruitment of SRC-1 to the transcription complex. These results show that TNF-α inhibition of vitamin D-action includes stable integration of p65 in the VDR transcription complex. Once anchored to proteins within the complex, p65 disrupts VDR binding to SRC-1, thus decreasing the efficiency of D-stimulated gene transcription. J. Cell. Biochem. 92: 833–848, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: vitamin D; TNF; VDR; NfκB; calcium; osteoporosis; transcription

Insufficiency of vitamin D causes reduced intestinal calcium absorption, parathyroid hyperplasia, and undermineralization of bone; changes that result in metabolic bone disease and fractures. Vitamin D insufficiency may result from a nutritional deficit, insufficient production of the active metabolite, 1,25-dihydroxyvitamin D3 (D), or resistance to D action as a consequence of vitamin D receptor (VDR) mutations or polymorphisms [Jurutka et al., 2000; Holick, 2003]. A more subtle but commonly acquired resistance to D occurs with menopause, when estrogen levels wane and the D dose response curve shifts rightward [Gennari et al., 1990; Koren et al., 1992; Liel et al., 1999; Pattanaungkul et al., 2000]. Decreased estrogen causes an increase in the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), a major contributor to the pathophysiology of skeletal disease [Cenci et al., 2000; Roggia et al., 2001]. Our laboratory has previously shown that TNF-α inhibits D action in the osteoblast, suggesting that increased TNF-α could contribute to D resistance after menopause [Nanes et al., 1991, 1994; Kuno et al., 1994; Farmer et al., 2000].

Using the skeletal-specific osteocalcin gene as a model of D-responsiveness, we showed that TNF-α inhibited D-stimulated osteocalcin gene transcription. Interestingly, the inhibition of

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D-stimulated transcription could not be attributed to a TNF-α-responsive region of the promoter, but was conferred by the vitamin D response element (VDRE) [Nanes et al., 1994]. These findings suggest that TNF-α impairs the transcriptional potency of the VDR. Indeed, TNF-α destabilizes binding of the VDR and its associated nuclear proteins to the osteocalcin promoter [Fernandez-Martin et al., 1998]. Farmer et al. [2000] reported that the inhibitory effect of TNF-α on D-stimulated transcription could be reproduced by forced expression of the TNF-α-activated transcription factor NfκB, or by expression of the p65, but not the p50, subunit of NfκB. Since p65 does not bind the VDR or the VDRE directly, we considered that p65 might interact with the transcriptional machinery downstream of the VDR, thereby inhibiting activation of a fully functional polymerase complex.

The ligand-activated VDR binds DNA in the form of a heterodimer with the retinoid X receptor (RXR). Ligand activation may promote release of transcriptional corepressors and binding of CBP/p300 and the p160 family of nuclear proteins, the DRIP/ARC complex, and several pre-initiation factors, all of which are shared between the VDR and NfκB [Rachez et al., 1998; Naar et al., 1999; Chiba et al., 2000]. We hypothesized that NfκB could modulate D-stimulated transcription by competing for nuclear coactivators. Sharing of limited nuclear coactivators would allow for regulatory cross talk between different signal pathways and a mechanism to further modulate the level of gene transcription.

Here, we show that the p65 subunit of NfκB stably integrates into the D-dependent nuclear transcription complex in vivo and exerts an inhibitory effect on D-stimulated transcription by disrupting the binding of VDR to SRC-1. Furthermore, structure-function studies reveal that two regions of p65 are required for this inhibitory activity, the N-terminal rel homology domain (RHD), and a midmolecular phosphoaminoacid region. The potency of D may be regulated by TNF-α through this NfκB dependent mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Assays

ROS 17/2.8 cells were plated at 1.2×10^5 cells per well in 12-well plates, or 2×10^6 cells per 75 mm flask, and grown in F-12 medium plus 10% fetal calf serum. For chromatin immunoprecipitation assays (CHIP), cells were treated with 10^{-8} M $1,25(OH)_2D_3$ (Biomol, Plymouth Meeting, PA), 10 ng/ml TNF-α (PeproTech, Rocky Hill, NJ), or both, in F-12 medium containing 1% fetal calf serum 2 days after plating. For transfection and luciferase studies, cells were treated with 10^{-8} M $1,25(OH)_2D_3$, 10 ng/ml TNF-α, or both, in F-12 medium containing 1% fetal calf serum 1 day after transfection. Where used, the addition of histone deacetylase (HDAC) inhibitors preceded treatment with $1,25(OH)_2D_3$ or TNF-α by 2 h. Transfections were performed using Superfect (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Cells were harvested with lysis buffer (Promega, Madison, WI) 24 h after treatment for measurement of luciferase or CAT activity. Luciferase activiy was measured using the Luciferase Assay System (Promega). Chloramphenicol acetyltransferase activity (CAT) was measured using a CAT Elisa Kit (Roche Applied Science, Indianapolis, IN). Transfection efficiency was corrected by cotransfection with the vector pSV40-β-gal (Promega). Quantitation of reporter activity was measured using a LumiCount luminometer (PerkinElmer, Boston, MA).

Plasmid Constructs and Antibodies

The vectors pRC-CMVp65 and pTK-NFkB-Luc were provided by Dr. M.L. Schmitz (Gent University, Gent, Belgium). The pCMX-ACTR (SRC-3) was provided by Dr. R.M. Evans (The Salk Institute, San Diego, CA). The pcDNA3-CBP was provided by Dr. K. Senger (Columbia University, New York, NY). The pCR3.1-hSRC-1a was provided by Dr. M.-J. Tsai (Baylor College of Medicine, Houston, TX). The (−522)OC CAT was provided by Dr. Marie Demay (Harvard Medical School, Boston, MA). The pTK-VDRE2-Luc was constructed by subcloning the VDRE fragment from (−522) OC CAT into pTK-Luc vector (Clontech, Palo Alto, CA). The pEF/myc/nuc-p65 was constructed by subcloning the p65 from pRC-CMVp65 into pEF/myc/nuc vectors (Invitrogen, Carlsbad, CA). The deletion mutants, p65Δ(18–167), p65Δ(193–289), p65Δ(1–17, 168–551), and p65Δ(1–192, 289–551) were originally made by QuikChange Site-Directed Mutagenesis kit (Stragene, La Jolla, CA) in the pRC-CMV vector using the following sets of primers: gcagagccagcccaggcctctccatcagg-

caggcccctccgc and gcggaggggcctgcctgatg gagaggcctgggctggctctgc; aatcgtgcccccaacactgccccagatacagacgatcgtcac and gtgacgatcgtctgtatctg gggcagtgttgggggcacgatt; agtgcaggcatgggcccctatgtggag, ctccacataggggcccatgcctgcact, acagtgcgggactagggtgacgcctggacctccc and gggaggtcaggcgtcaccctagtcccgcactgt; tgtagtgcaatggagctcaagatctgc, gcagatcttgagctccattgcactaca, ttccagtacctgtagggtgacgcctgacctccc and gggaggtcaggcgtcaccctacaggtactggaa, respectively. The p65Δ(18–167) and p65Δ(193–289) were subsequently subcloned into the vector pEF/myc/nuc. The forward primers, ggcttcctcgaggctgagctc, gccgagctcgagatctgccga, gagcccctcgagttccagtac, acgctgctcgaggccctgctg and reverse primer, aggggcggccgcacccccttcg were used for PCR and cloning p65Δ(1–100), p65Δ(1–193), p65Δ(1–286), p65Δ(1–306), and p65Δ(1–438) correspondingly, all of which utilized pEF/myc/nuc vector's atg as the starting codon. The forward primer, ggccatggacgaactgttc and reverse primers, gtcggcggccgcacgctgctcttc, ggtcgcggccgcaatggccac, gatgctcttggcggccgcat atgtcct, atagggcgcg gccgctggcttg, and cagggcggcc gcatcaaactgc were using for PCR and cloning pEF/myc/nuc p65Δ(149–551), p65Δ(250–551), p65Δ(308–551), p65Δ(344–551), p65Δ(446–551), respectively. The primer set, ctgcggcggcctgccgaccgggag and ctcccggtcggcaggccgccgcag; gagctcgctgagcccatggaattc and gaattccatgggctcagcgagctc; cggacccctcccgccgcagaccccagc and gctggggtctgcggcgggagggtccg; atggaattccaggccctgccagatacaand tgtatctggcagggcctggaattccat were used to make pEF/myc/nuc p65-S276A, p65-S281A, p65-Y257A, and p65-Y288A by QuikChange Site-Directed Mutagenesis kit. Dideoxynulceotide sequencing was done to confirm the final vector sequence using an ABI Prism™ dye terminator cycle sequencing ready kit (PerkinElmer, Wellesley, MA) on an Applied Biosystem 313 automated DNA sequencer. The pGEX2t VDR-LBD was provided by J. White (McGill University, Montreal, Canada). Antibodies used in the experiments included anti-cMyc and an anti-C-terminal- and anti N-terminal-p65 (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vitro Binding Reaction

Proteins studied in pull-down assay were synthesized in vitro in the presence of S^{35}-methionine (Amersham, Piscataway, NJ) using the TNT Quick Coupled transcription and translation system (Promega) according to the manufacture's recommendations. The p65 full-length and its deletion or mutation mutants were synthesized similarly but using nonradioactive methionine. One to 20 μl of the reaction products, as indicated for each experiment, were mixed with either glutathione S-transferase (GST) protein or GST-VDR-LBD in 500 μl (total volume) of binding buffer (20 mM Tris-HCL, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5% NP40, 0.5% BSA) in the presence of GST beads (Sigma, St. Louis, MO), and incubated at 4°C with gentle rocking for 1 h. Beads were then washed three times with the binding buffer, resuspended in SDS loading buffer, boiled and analyzed on SDS–10% polyacrylamide gels. The gels were dried and exposed against an intensifying screen and scanned using a Storm 860 (Amersham) and quantified using the manufacturer's ImageQuant 5.1 software.

Immunoprecipitations and Western Blot Analysis

Nuclear proteins from transfected cells were harvested and lysed using NE-PER Nuclear Extraction Regents (Pierce, Rockford, IL) according to the manufacture's recommendations. The nuclear extracts were incubated with the c-Myc antibody in a total volume of 500 μl IP buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and 1 μg each of aprotinin, leupeptin, and pepstatin/ml) for 2 h at 4°C. Fifty microliters Protein A agarose beads were added and rotated for another 1 h. The complexes were then centrifuged at 4°C for 15 s and the beads were washed three times with 1 ml IP buffer. Beads were resuspended in SDS loading buffer, heated at 70°C for 5 min, and analyzed on SDS–10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Invitrogen), blocked with 5% dry milk in PBS-T (0.3% Tween 20 with 1× phosphate buffered saline), and incubated with antibodies to N- or C-terminal p65 or cMyc for 1 h. Membranes were washed with PBS-T and incubated for 1 h with secondary antibody conjugated to horseradish peroxidase (Santa Cruz), washed again ×5 with PBS-T, and incubated with chemiluminescence regent (Pierce). Membranes were exposed to X-ray film to visualize the bands.

ChIP Assays

ChIP assays were preformed using a ChIP Assay Kit (Upstate Biotechnology, Lake Placid,

NY) under modified conditions. 2×10^6 cells were treated with D or TNF-α, as described for each experiment, and fixed with 1% formaldehyde for 15 min. Cells were washed two times with ice-cold PBS and lysed for 10 min in lysis buffer (Upstate Biotechnology). Cross-linked chromatin-DNA-protein complexes were sheared by sonication and pre-cleared for 2 h at 4°C with salmon sperm DNA saturated protein G Sepharose. Chromatin solutions were precipitated 1 h at 4°C using 2 μg anti-p65, anti-SRC-1, or mouse IgG. Immune complexes were collected with salmon sperm DNA-saturated protein G Sepharose for 1 h and washed extensively following the manufacture's protocol. Chromatin solutions were then incubated at 70°C overnight to reverse cross-links. DNA was extracted with phenol/chloroform and precipitated with ethanol and analyzed by PCR (35 cycles) using *Taq* PCR Master Kit (Qiagen). The following primer sets were used: tgtctctgacacaaacagggc and gagcggtcagtaatgtcctgattcacc, to amplify a 341 base-pair region of VDRE (vitamin D3 enhancer) on the rat osteocalcin (OC) promoter; gatatcaagtgggctcctac and gcgggttacgtatttcagag, to amplify a 202 bp fragment of a non-specific repeat region on the rat OC promoter as one control, and aacccagaattacctgatcc and gaagcatatagtagcgtcaacc to amplify a 303 bp fragment of a region in the osteocalcin coding sequence over 1 kb downstream of the VDRE as a second control. The PCR products were separated in 2% agarose gels containing 0.5 μg/ml ethidium bromide. DNA bands were visualized using ultraviolet light and recorded as tagged image file format (TIF) using UVP BioDoc-It System (UVP, Upland, CA). TIF files were subjected to densitometric analysis by ImageQuant 5.1 software. For the p65 or empty vector transfection, cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle control. Additional ChIP assays were done similarly substituting antibodies other than p65 as indicated.

RESULTS

p65 Co-Occupies the Vitamin D Transcription Complex

To determine if p65 could bind to the D-stimulated transcription complex in vivo, we performed ChIP in osteoblastic ROS 17/2.8 cells. Cells were treated with control media, D (10^{-8} M), TNF-α (10 ng/ml), or TNF-α + D; and the resulting chromatin-DNA-protein complexes were cross-linked as described in "Experimental procedures." p65-bound protein-DNA complexes were immunoprecipitated with p65 antibody and quantitation of bound complexes was done by PCR using primers flanking the VDRE (−773/−433), an upstream control region (−1377/−1176), and a distal downstream control region in the coding sequence (+999/+1302). Figure 1 shows results of a ChIP assay for the signal linked to immunoprecipitated p65. Figure 1 (top) shows a map of the osteocalcin promoter and PCR primer locations. Figure 1A, left panel, shows that the PCR signal from cells treated with TNF-α + D is increased relative to signals from control, D, or TNF-α treated groups. The right panel in Figure 1A shows the mean ± SEM signal quantitation from three identical experiments. Amplification of the −1377/−1176 control promoter region (Fig. 1B, left panel) revealed a weak background signal but failed to show a difference in signal intensity between treatment groups. In addition, amplification of the downstream control sequence 999/1302 in the coding region did not detect a signal despite adequate input (Fig. 1C, left panel). These results confirmed the specificity of p65 integration into the VDRE-bound complex. The right panels for Figure 1B and C, shows the mean ± SEM results from three such ChIP experiments. No signal was obtained using rabbit IgG in place of the p65 antibody, as expected (not shown).

Figure 2 shows results of ChIP assays done as in Figure 1, except that ROS 17/2.8 cells were transiently transfected with a constitutive p65 expression vector (pRC-CMVp65) in place of TNF-α treatment. Figure 2A, left panel, shows the PCR signal for the VDRE from cells treated with a control empty vector (C), D + empty vector (D), p65 vector (p65), or D + p65. An increase in signal intensity is seen in the D + p65 group. This relative difference in intensity was not observed using primers that flanked the distal repeat region (−1377/−1176) or the downstream control region (+999/+1302), as seen in Figure 2B and C. Graphs in the right panel of Figure 2A and B show quantitation of the signal from three experiments.

RHD of p65 Is Required for Inhibition of VDR Activity

Since the ChIP assays suggested that p65 could integrate directly into the VDR transcription

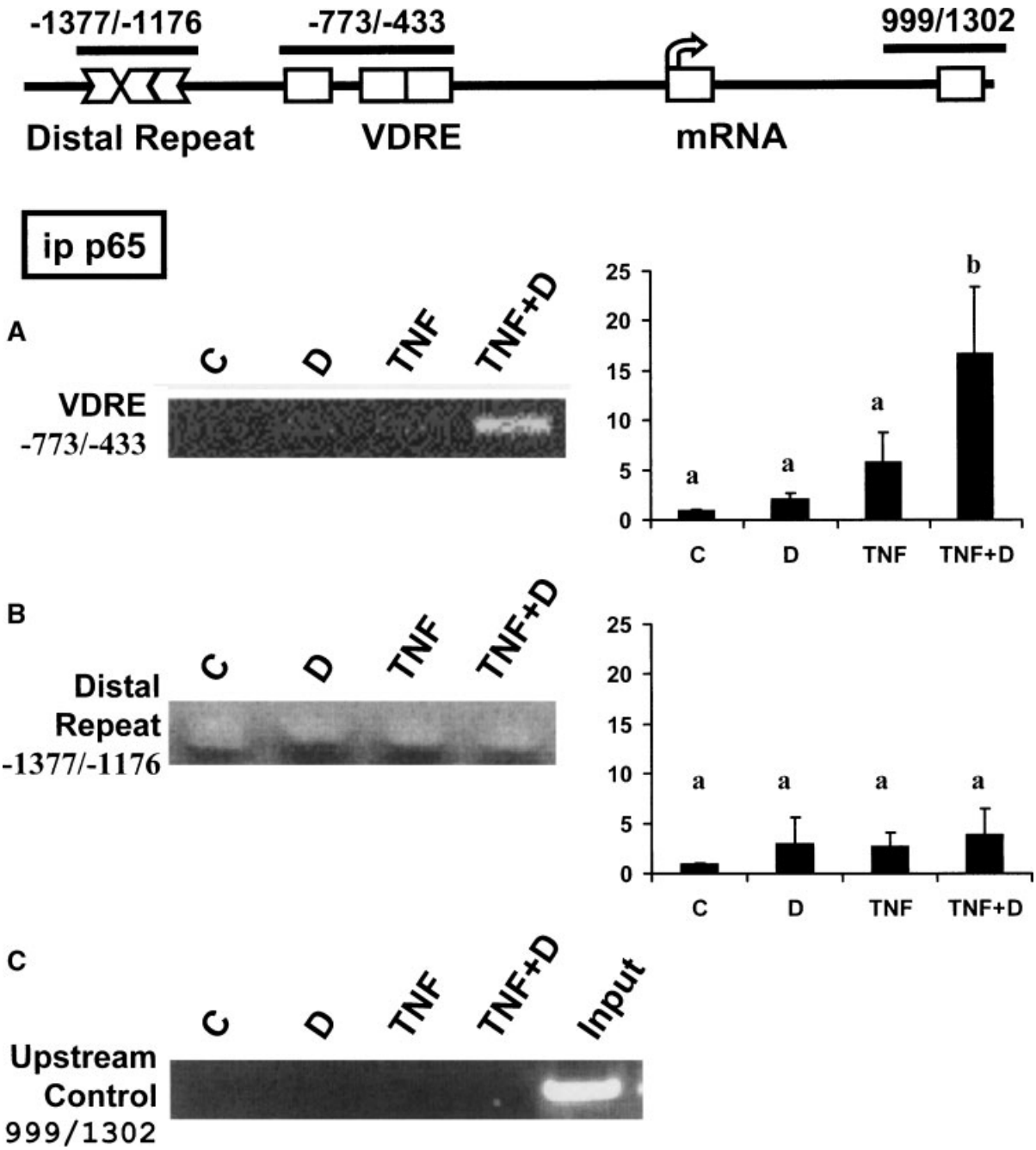

Fig. 1. TNF-α stimulates integration of p65 into the vitamin D transcriptional complex on the osteocalcin promoter. Chromatin immunoprecipitation assays (ChIP) assay primer locations are depicted at top. Immunoprecipitation of cross-linked protein-DNA complexes was done with antibody to p65 (ip p65). **Left panel**: ChIP PCR signals for a representative experiment. **Right panel**: Mean ± SEM signal quantitation for three experiments. **A**: ChIP assay PCR signal for protein/DNA complex containing p65 on the vitamin D response element (VDRE). **B**: ChIP assay PCR signal for protein/DNA complex on an upstream control promoter sequence, −1377/−1176, showing background signal. **C**: ChIP assay PCR signal for protein/DNA complex on a downstream control coding sequence, +999/+1302, showing no signal. The input control for the downstream sequence was obtained by PCR of the pre-immunoprecipitated samples to confirm sample loading. C (control); D (10^{-8} M $1,25(OH)_2D_3$); TNF-α (10 ng/ml). Bars with different letters are significantly different from each other by ANOVA, $P < 0.05$.

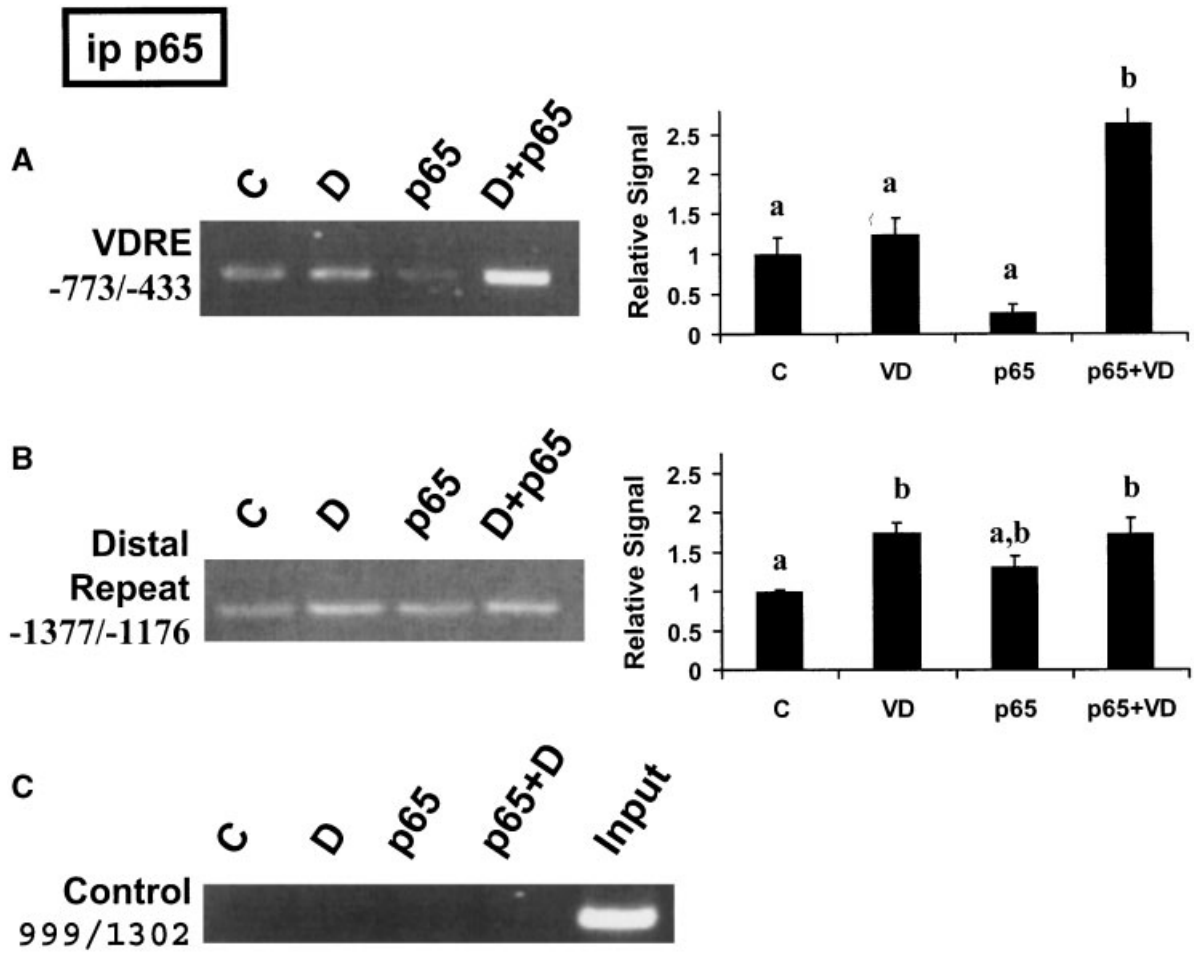

Fig. 2. Integration of p65 into the vitamin D transcriptional complex on the osteocalcin promoter after transfection with p65 vector. Immunoprecipitation of cross-linked protein-DNA complexes was done with antibody to p65 (ip p65). **Left panel**: ChIP PCR signals for representative experiments. **Right panel**: Mean ± SEM signal quantitation for three experiments. **A**: ChIP assay PCR signal for protein/DNA complex containing p65 on the VDRE. **B**: ChIP assay PCR signal for protein/DNA complex on an upstream control sequence, −1377/−1176, showing background signal. **C**: ChIP assay PCR signal for protein/DNA complex on a downstream control sequence, +999/+1302, showing no signal. The input control for the downstream sequence was obtained by PCR of the pre-immunoprecipitated samples to confirm sample loading. C (control, empty expression vector); D (10^{-8} M $1,25(OH)_2D_3$ + empty expression vector); p65 (pRC2CMV-p65 expression vector). Bars with different letters are significantly different from each other by ANOVA, $P < 0.05$.

complex, we sought to determine the functional domains of p65 that were required for inhibition of VDR transactivation. A transcription reporter was constructed by inserting two copies of the rat osteocalcin VDRE upstream of a minimal herpes simplex virus thymidine kinase promoter controlling expression of luciferase. The effect of p65 on D-stimulated transcription of the test reporter is shown in Figure 3A. D stimulates transcription of 2× VDRE-LUC 16-fold and co-transfection of 100 ng of pRC-CMVp65 inhibits the response to D by 50%. Figure 3B and C show similar experiments using a fragment of the rat osteocalcin promoter (−522OC-CAT) for comparison. As previously shown, vitamin D stimulates a 2.5-fold increase in −522OC-CAT activity, which is reduced by simultaneous treatment with TNF-α (Fig. 3B) or the p65 expression vector (Fig. 3C).

p65 deletion mutants were expressed using the EF-1a promoter-driven expression plasmid, pEF/myc/nuc, which includes an independent SV40-large T antigen nuclear localization signal and a c-myc epitope tag for detection ("Experimental procedures"). Western analysis of nuclear extracts from ROS 17/2.8 cells that had been transfected with wild type or mutant

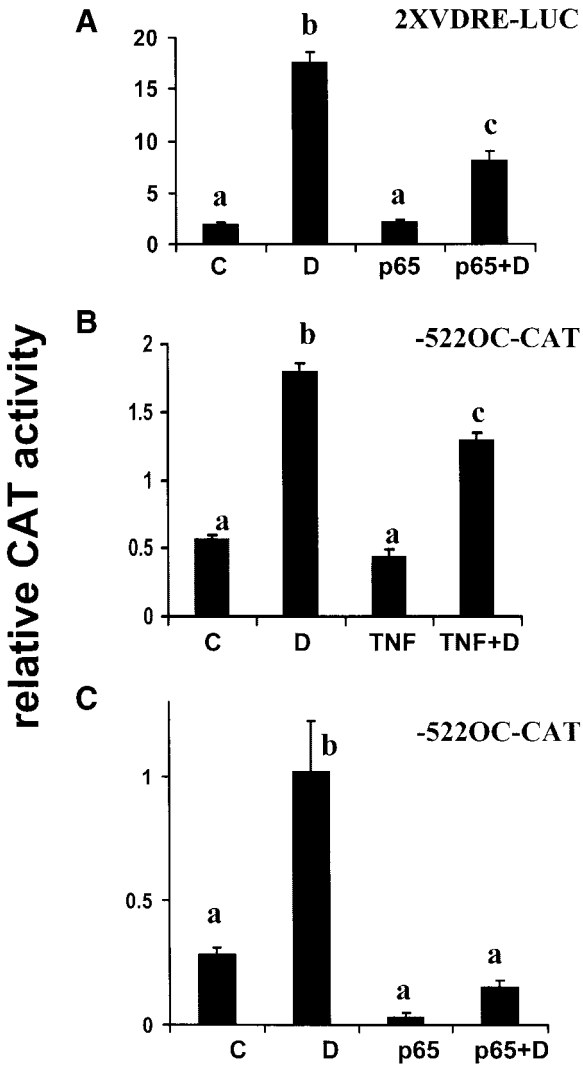

Fig. 3. Effect of TNF-α or p65 expression on transcription of a heterologous VDRE-reporter or a VDRE-containing osteocalcin promoter fragment. Results show mean ± SEM arbitrary transcription units in ROS 17/2.8 cells. The vitamin D-responsive reporter activity was corrected for transfection efficiency using an SV40β galactosidase reporter. **A**: Cells were transfected with 2× VDRE-LUC as described in "Experimental procedures" and 24 h later treated with C (empty vector); D (10^{-8} M $1,25(OH)_2D_3$ + empty vector); p65 (p65 expression vector). Cell lysates were obtained for luciferase and β galactosidase assays 24 h after treatment. **B**: As in (A) but using the CAT reporter containing the osteocalcin promoter −522 bp fragment and treatment with TNF-α instead of p65. C (control); D (10^{-8} M $1,25(OH)_2D_3$); TNF-α (10 ng/ml). **C**: As in (A) but using −522OC-CAT reporter. Bars with different letters are significantly different from each other by ANOVA, $P < 0.05$.

p65 expression plasmids revealed the expected sized band for each construct 24 h after transfection. A representative Western analysis of expressed p65 wild type and mutants is shown in Figure 4A. Figure 4B and C shows experiments in which the inhibitory effect of each mutant was assessed relative to D stimulation. Here, D stimulation of transcription is plotted arbitrarily as 1.0. Several deletions encompassing the RHD or substitutions of midmolecular serines and tyrosines abolished p65 activity. Figure 5 summarizes the mutations of p65 averaged over multiple experiments as they relate to a p65 functional domain map (Fig. 5, Top). In the left side of Figure 5, dotted lines denote the deleted regions of p65 or the location of neutral amino acid substitution of serines or tyrosines. The right side of Figure 5 shows the inhibitory potency of the mutated p65 on vitamin D-stimulated transcription expressed as a percentage of p65 wild type activity. The data reveals that the inhibitory potency of p65 is lost with deletions that disrupt the RHD near the N-terminus of the protein. In addition, loss of p65 activity is seen with deletions that encompass or are proximal to several midmolecular serine or tyrosine residues (S281A, Y257A). These residues were selected for study because their flanking sequences were found to predict phosphoserine or phosphotyrosine sites [Blom et al., 1999]. No inhibitory effect of p65 on vitamin D-stimulated transcription was observed using two small peptides that included only the RHD or the midmolecular region, suggesting that both are needed for full potency (not shown).

To determine if the p65 domains contributing to D-inhibition are similar to those functioning in NfκB transcriptional activation, we studied the effect of p65 deletions on an NκB-responsive reporter (NfκB-LUC) (Fig. 6). Transfection of p65 stimulates this reporter 26-fold. The N-terminal, midmolecular, and C-terminal deletions all diminished p65 transcriptional potency. Of note, p65Δ344–51, which maintained 67% of wild type activity for D-inhibition, almost completely abolished p65 transcriptional activity.

Repression of VDR Transcription by p65 Is not Prevented by Inhibition of Histone Deacetylases

Since NfκB can bind the histone deacetylases, HDAC1 and HDAC2, we evaluated the possibility that recruitment of these HDACs by p65 contributed to inhibition of transcription. To test this hypothesis we used two HDAC inhibitors, sodium butyrate (NaButr) and trichostatin A (TSA). D responsiveness was measured in ROS 17/2.8 cells that were transiently

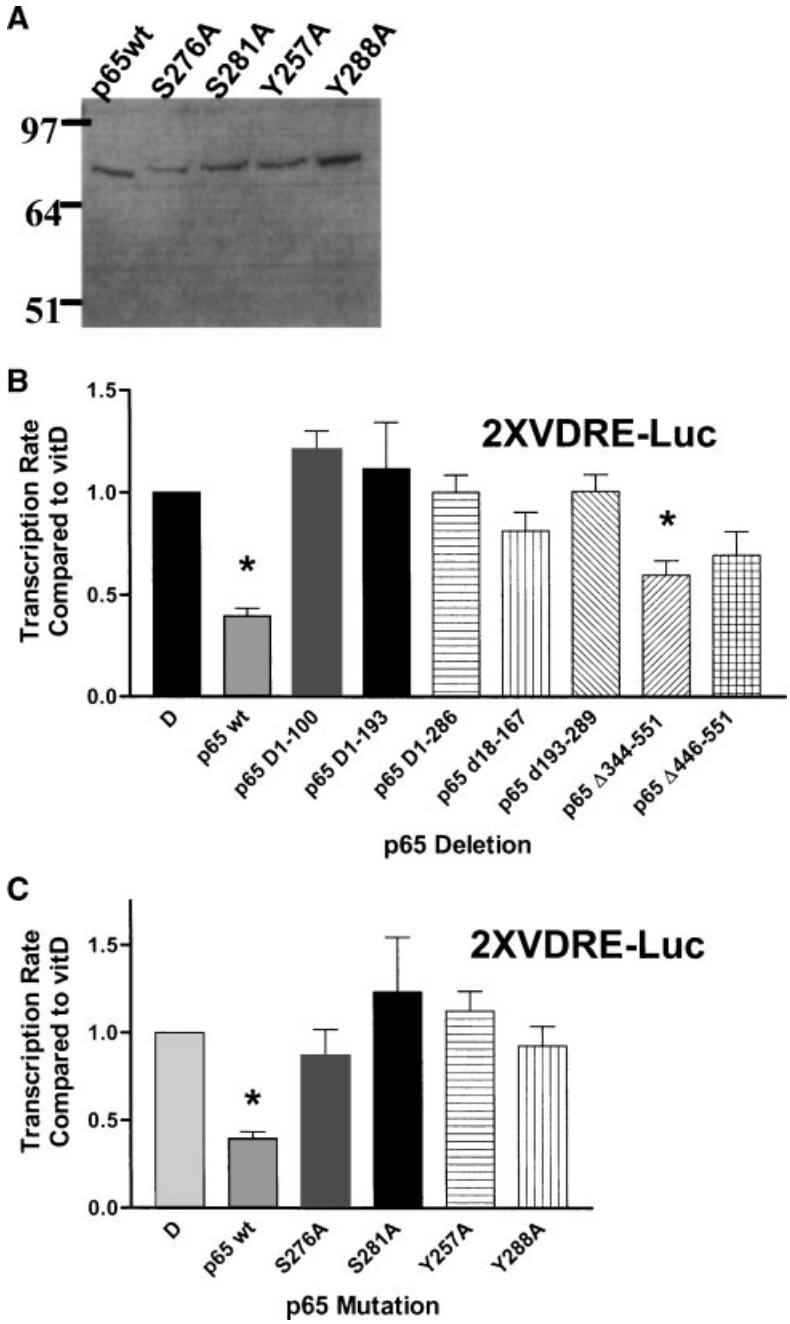

Fig. 4. Effect of p65 mutations on inhibition of vitamin D-stimulated transcription. ROS 17/2.8 cells were transfected with the 2× VDRE-LUC reporter and pEF/myc/nuc vectors containing wild type or mutant p65 and treated with 10^{-8} M $1,25(OH)_2D_3$. Results show the mean ± SEM transcriptional units after 24 h for each p65 mutant relative to treatment with vitamin D alone (set to 1.0 arbitrary units). All transcription assays were corrected for efficiency of transfection using an SV40βgalactosidase reporter. **A**: Western analysis of transfected ROS cells showing myc-tag detection of the expressed p65 wild-type and representative mutants. **B**: Effect of representative deletion mutants of p65. **C**: Effect of point mutations of p65 in the midmolecular region. *, $P < 0.05$ versus D by ANOVA.

Fig. 5. Domains of p65 that inhibit vitamin D-stimulated transcription. Summary of pooled results from deletion mutants and neutral amino acid substitutions in which the effect of p65 on vitamin D-stimulated transcription was determined as in Figure 5. A map of p65 indicates the known functional domains and regions of phosphorylation (hatched area) is shown at top. **Left column**: Map of deletions and substituted phosphoaminoacids; **middle column**: Amino acid limits of the mutations; **right column**: Inhibitory potency of each mutant as percentage of wild type p65.

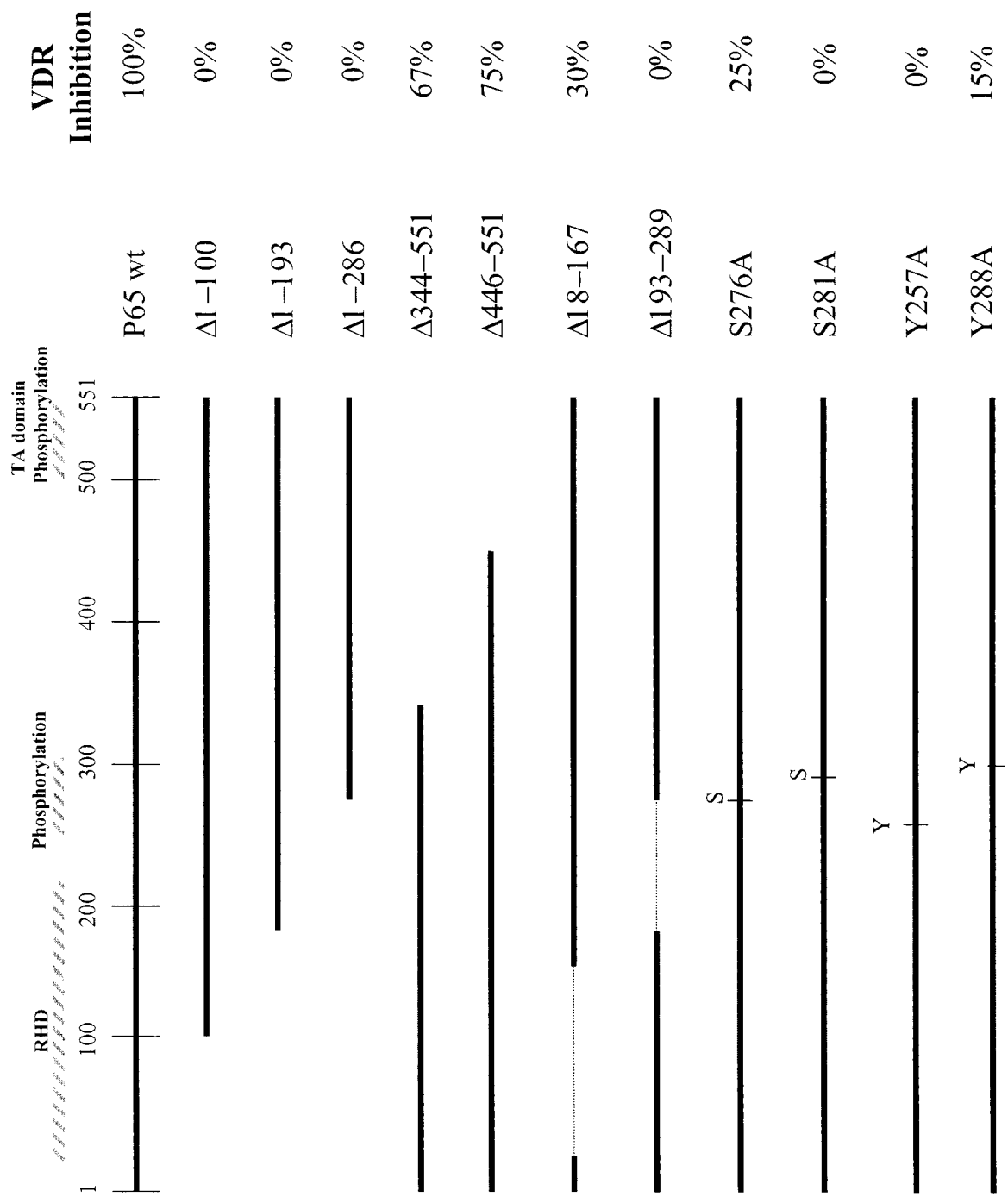
RHD
Phosphorylation
TA domain
Phosphorylation
1
100
200
300
400
500
551
S
S
Y
Y
VDR Inhibition
P65 wt 100%
Δ1–100 0%
Δ1–193 0%
Δ1–286 0%
Δ344–551 67%
Δ446–551 75%
Δ18–167 30%
Δ193–289 0%
S276A 25%
S281A 0%
Y257A 0%
Y288A 15%

Fig. 5.

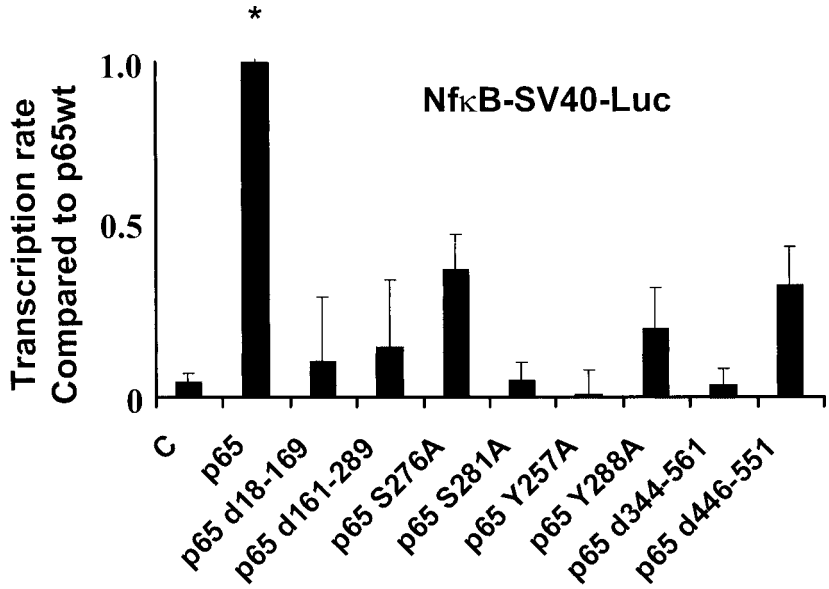

Fig. 6. p65 mutations diminish NfκB transcriptional potency. Ros 17/2.8 cells were transfected with the NfκB-TK-LUC reporter and wild type or mutant p65 expression vectors. Results show mean ± SEM transcriptional stimulation 24 h after transfection relative to wild-type p65, which is set arbitrarily to 1.0. The absolute stimulation by wild-type p65 was 2.6-fold. All mutations are significantly diffeent from wild type p65, $*P < 0.05$ by ANOVA.

transfected with the VDRE-LUC reporter, with or without the p65 expression vector. The effect of HDAC inhibitors on p65 action is shown in Figure 7. When compared with D-stimulated transcription, p65 inhibits the vitamin D response by 50%, as shown previously. Both HDAC inhibitors increased basal and D-stimulated transcription; however, p65 continued to suppress the D response by 50% when compared to the respective HDAC control.

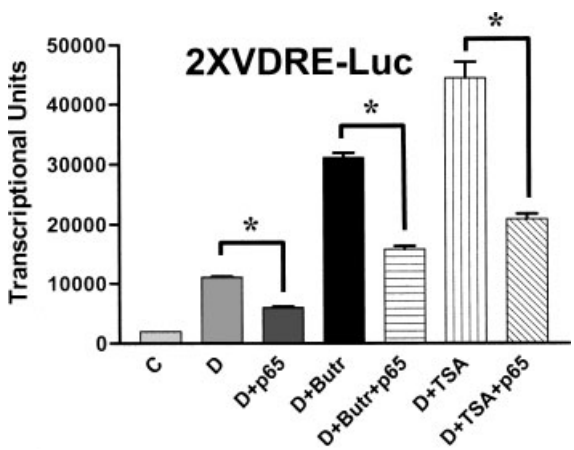

Fig. 7. Histone deacetylases do not prevent p65 inhibition of vitamin D action. ROS cells were transfected with a 2× VDRE-LUC reporter and the effect of p65 on D-stimulated transcription was assessed in the presence or absence of HDAC inhibitors as described in "Experimental procedures." The results show mean ± SEM arbitrary transcriptional units 24 h after treatments. C (control, empty vector); D (10^{-8} M $1,25(OH)_2D_3$ + empty vector); p65, (p65 expression vector); Butr (sodium butyrate), TSA (trichostatin A). $*P < 0.05$ by ANOVA.

p65 Inhibits VDR Binding to SRC-1

To determine if p65 interfered with binding of the VDR to CBP or SRC-1, we carried out pull-down assays in which in vitro synthesized ^{35}S-CBP or ^{35}S-SRC-1 binding to the VDR could be measured. Binding of ^{35}S-CBP to GST-VDR ligand-binding domain was not observed using pull-down assays (not shown). Figure 8A shows that a weak binding of SRC-1 to VDR is observed in the absence of D (lane 3). Addition of D increases VDR-SRC-1 binding as seen in lane 2. The signal is absent without addition of VDR (GST only) (lane 1). Addition of wild type p65 diminishes D-stimulated binding of SRC-1 to VDR (lane 4). The ^{35}S-SRC-1 input for these assays is shown in lane 8. p65 mutants that failed to inhibit D-stimulated transcription were also studied in the pull-down assay. It can be seen that the addition of the p65 mutants Δ18–169, Δ161–289, and Y288A failed to inhibit SRC-1 binding to the VDR (lanes 5–7) when compared to the effect of wild type p65 (lane 4). These results are concordant with the

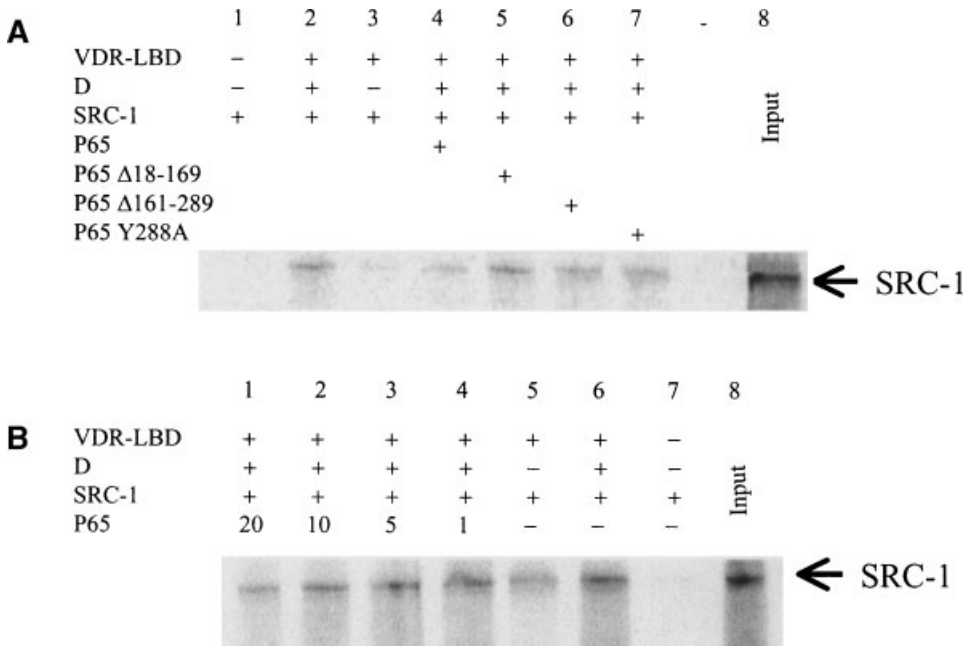

Fig. 8. p65 inhibits VDR-SRC-1 binding. Pull-down assays using GST-VDR-ligand binding domain (VDR-LBD) immobilized on glutathione sepharose beads. ^{35}S-SRC-1 is added to the GST-VDR ± D (1,25(OH)$_2$D$_3$, 10^{-8} M). **A**: Binding of SRC-1 to the VDR is stimulated by D (**lane 3** vs. **lane 2**). Addition of wild-type p65 inhibits VDR-SRC-1 binding (**lane 4** vs. lane 2). Inhibition is not observed with addition of mutant p65 (**lanes 5**, **6**, or **7** vs. 2). **Lane 8**, SRC-1 input. **B**: p65 inhibition of VDR-SRC-1 binding is dose dependent. Total protein added to the binding reaction was held constant by adding control translation mix (no p65 plasmid). Lane 8, SRC-1 input.

loss of inhibitory potency of these mutations in the transient transfection assays.

To determine if the inhibitory effect of p65 on VDR-SRC-1 binding is dose dependent, increasing amounts of p65 were added to the reaction mix in the pull-down assay. Figure 8B shows that the inhibitory effect of wild type p65 increases as a greater amount of p65 protein is added.

TNF-α Prevents D Recruitment of SRC-1 to the Transcription Complex

ROS 17/2.8 cells were treated with vitamin D and co-transfected with CMV-driven vectors for p65, SRC-1, p65 + SRC-1, or an empty control vector. The transcriptional response of the 2× VDRE-LUC reporter was then measured. Figure 9A shows the expected inhibition of vitamin D-stimulated transcription by p65. Expression of SRC-1 increased D-stimulated transcription; however, p65 continued to inhibit vitamin D action. Similar experiments using either CBP or the SRC-1 homologue, SRC-3 (ACTR, pCIP, RAC3, AIB1, TRAM-1), were also done to determine if their expression could overcome p65 inhibition. As seen in Figure 9B and C, neither CBP nor SRC-3 prevented p65 inhibition of transcription. In addition, expression of DRIP205, a member of the DRIP/ARC complex that binds VDR directly, did not prevent p65 inhibition (not shown).

ChIP assays were done to determine if TNF-α treatment was displacing SRC-1 from the complex. Figure 10 shows the results of an experiment in which cells were treated with D, TNF-α, or TNF-α + D, followed by ChIP assay using a SRC-1 antibody for immunoprecipitation. In this experiment, the SRC-1 signal was increased by overexpressing SRC-1 as in Figure 9A. The PCR signal generated using primers flanking the VDRE revealed D-stimulated increase in SRC-1 association (lane 2 vs. lane 1), as expected. SRC-1 recruitment to the complex was diminished in cells treated with D + TNF-α (lane 4 vs. lane 2).

DISCUSSION

Cross talk between intracellular signal pathways provides an important modulation of the rate of gene transcription. We, and others, have previously shown that TNF-α inhibits the rate of gene transcription by ligand activated nuclear hormone receptors [Nanes et al., 1991;

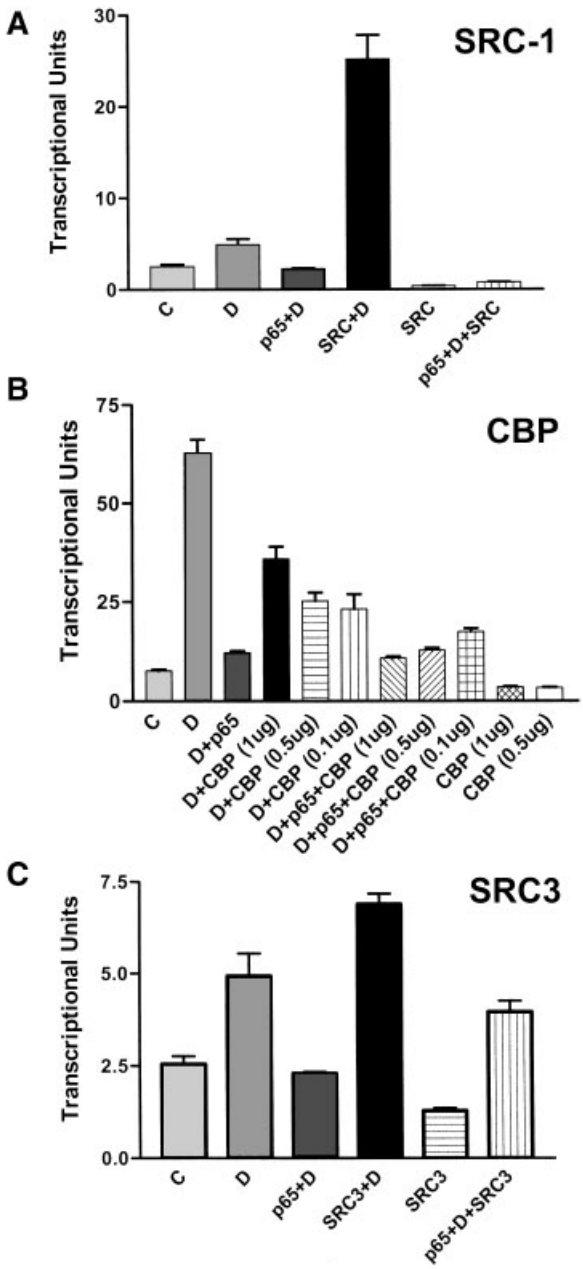

Fig. 9. Overexpression of coactivators SRC-1, CBP, or SRC-3 does not prevent p65 inhibition of D-stimulated transcription. ROS 17/2.8 cells were transfected with the 2× VDRE-LUC reporter and p65 expression vector as in Figure 5. The effect of the p65 on D-stimulated transcription was then measured in cells cotransfected with SRC-1, CBP, or SRC-3 as indicated in the figures **A**, **B**, or **C**, respectively. p65 inhibited the transcriptional response to D regardless of coactivator overexpression.

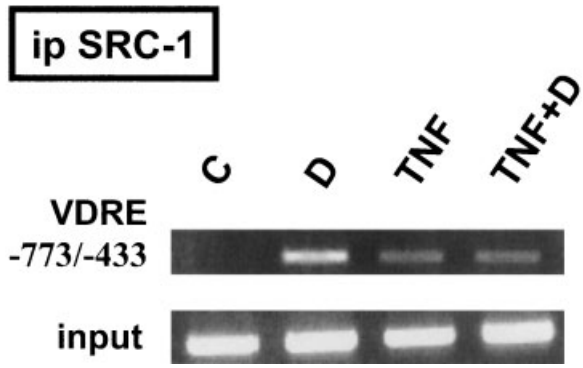

Fig. 10. TNF-α prevents D-stimulated recruitment of SRC-1 to the transcriptional complex on the osteocalcin promoter VDRE. Immunoprecipitation of cross-linked protein-DNA complexes was done with antibody to SRC-1 (ip SRC-1). **Top panel**: ChIP PCR signals for a representative experiment. **Bottom panel**: Input PCR signals for samples from each group. SRC-1 signal is weak in untreated control cells (**lane 1**) but increases after treatment with D (**lane 2**). TNF-α + D signal diminishes the D-stimulated recruitment (**lane 4**). C (control); D (10^{-8} M $1,25(OH)_2D_3$); TNF-α (10 ng/ml).

Kuno et al., 1994; Caldenhoven et al., 1995; Scheinman et al., 1995; Supakar et al., 1995; Palvimo et al., 1996; van der Saag et al., 1996; De Bosscher et al., 1997; Fernandez-Martin et al., 1998; McKay and Cidlowski, 1998; Sheppard et al., 1998; Pelzer et al., 2001; Nelson et al., 2003]. For members of the vitamin D/thyroid/retinoid family of nuclear receptors, ligand binding of the receptor induces a conformational change that promotes coactivator binding and consequent accessibility to components required for a functional RNA polymerase [Rachez et al., 2000]. Our data shows that the TNF-α-activated transcription factor, NfκB, can be integrated into the VDR transcription complex. Using ChIP assays, we show that the p65 subunit of NfκB binds to the D-stimulated nuclear protein complex assembled around the VDRE of the osteocalcin promoter. Once integrated, p65 blunts the magnitude of D-stimulated transcription. The increased ChIP signal for p65 was observed only after D + TNF-α or D + p65 treatment, but not after treatment with TNF-α or p65 alone, suggesting that p65 cannot bind the transcription complex on the promoter until after D stimulation of the VDR. Results from our ChIP assays for p65 were specific for the VDRE, since amplification of upstream or downstream regions of the promoter failed to reveal an enhanced signal.

Deletion analysis of p65 revealed that the N-terminus of p65 (RHD) and a midmolecular region are both needed for the p65 inhibition of D action. Consistent with this conclusion, expression of a fragment spanning only the midmolecular region was insufficient to confer activity. In addition, neutral amino acid sub-

stitution of several midmolecular serines or tyrosines abolished p65 activity. Previous reports studying p65 transcriptional potency on TNF-α-responsive genes revealed a role for S276, in this midmolecular region, and S536, in the C-terminal "TA" domain [Zhong et al., 1998; Sakurai et al., 1999; Okazaki et al., 2003; Vermeulen et al., 2003; Yang et al., 2003; Jiang et al., 2003a,b]. These serines are subject to phosphorylation by numerous kinases. Recent study using p65 null embryonic fibroblasts has shown that p65 stimulation of gene transcription and the anti-apoptotic function of NfκB requires S276, confirming the critical role of S276 in NfκB function. Our data supports a role for the midmolecular serines and tyrosines, including S276 in the inhibition of D action. We found that C-terminal deletion of amino acids 344–551 reduced p65 inhibition of D action but did not abolish it, suggesting that the midmolecular and N-terminal regions may be more important for this activity. p65 transcirptional activity was almost completely abolished by deletion of this region. Thus, we observed some differences in the domains of p65 that contribute toward D-inhibition and NfκB transactivation although most overlap. Although slight differences in expression between p65 constructs were observed between experiments, these differences were unlikely to account differences in activity when averaged over multiple experiments. Indeed, the mutant p65 constructs were sometimes expressed at a slightly higher concentration than wild type yet still had no activity. Our data does not directly address whether phosphorylation of S276 or the other midmolecular serines or tyrosines is required for the inhibition of D action. In addition, our constructs were not informative for the role of S311, recently shown to contribute to p65 transcriptional function and to be an important PKC_{ζ} target [Duran et al., 2003].

Several mechanisms have been proposed to explain transcriptional cross talk between NfκB and nuclear hormone receptors, including direct blockade of transcription factor binding to DNA, competition for coactivators, and interference with phosphorylation of Polymerase II [Sheppard et al., 1998; An et al., 1999; Nissen and Yamamoto, 2000; Speir et al., 2000]. We previously excluded direct binding of p65 to the VDRE or to the VDR/RXR dimer as mechanisms of transcription inhibition [Farmer et al., 2000]. Since the p65 subunit of NfκB provides transactivation function through interaction with coactivators, an effect on the availability or function of a coactivator shared by the VDR is likely [Schmitz and Baeuerle, 1991; Schmitz et al., 1994; Vermeulen et al., 2002]. We considered two models of coactivator inhibition by TNF-α: in the first, TNF-α-stimulated p65 acts as a simple competitive inhibitor for limiting amounts of nuclear coactivators, thus depleting the VDR-bound complex of essential components. Such a simple competitive inhibition would occur if p65 bound SRC-1 directly. In addition, the overexpression of SRC-1 would be expected to overcome p65 inhibition; however, neither direct p65-SRC-1 binding nor rescue by SRC overexpression was observed (although SRC-1 overexpression did maintain the basal D stimulatory effect on transcription). In the second model, p65 stably integrates into the VDR-bound complex, possibly anchored by coactivators other than SRC-1. In this model, the integrated p65 could block SRC-1 binding to the VDR indirectly, through a steric or charged hindrance, thus preventing rescue in our experiments by overexpressed SRC-1. In support of this hypothesis, ChIP assays revealed that TNF-α treatment prevented D-stimulated recruitment of SRC-1 to the complex. Our model is also consistent with previous reports showing that p65 does not bind SRC-1 directly but does bind CBP [Na et al., 1998]. Interestingly, phosphorylation of S276, has been shown to be required for p65 binding to CBP [Okazaki et al., 2003]. S276 mutation, in our hands, abolished p65 inhibition of D-stimulated transcription.

Our pull-down assays reveal that p65 disrupts SRC-1 binding to the ligand binding domain of the VDR in vitro. The VDR binds SRC-1 in a ligand-dependent manner, similar to other nuclear hormone receptors. In addition, mutants of p65 that reduced its inhibitory effect on transcription in transient transfection studies failed to inhibit VDR-SRC-1 binding. We were unable to show binding of ^{35}S-p65 directly to GST-VDR ligand binding domain or full-length VDR in pull-down assays, confirming our previous results using electrophoretic mobility shift assays, and only very weak binding of ^{35}S-SRC-1 to GST-p65. These results suggest that a simple competition between these proteins is not operating; rather, p65 may anchor to other proteins, such as CBP, as discussed above and considered previously [Dominguez et al., 2001]. Although our present study focused on the

general coactivators of transcription, CBP/p300 and p160 proteins, we recognize that other nuclear proteins are required for optimal transcriptional activation by the VDR and further study will be needed to address each as possible targets of p65.

In our investigation we also considered that p65 might recruit a histon deacetylase to the VDR complex since NfκB is known to associate with HDACs [Ashburner et al., 2001; Zhong et al., 2002]. We found that treatment of cells with the HDAC inhibitors Na-butyrate or TSA increased basal D-stimulated transcription, as expected, but did not prevent p65 from inhibiting transcription. These results suggested that p65 did not recruit a histone deacetylase as part of the inhibitory mechanism.

Three-dimensional models have been solved for NfκB family proteins bound to DNA and to cytosolic IκB [Chen et al., 1998; Huxford et al., 1998; Menetski, 2000; Escalante et al., 2002; Malek et al., 2003]. In these models, the midmolecular serines and tyrosines critical for the inhibitory cross talk with the VDR are exposed at the periphery of the complex where they are potentially available to provide steric or charged interference between the VDR and SRC-1. Further testing will be needed to address this possibility. Our present study reveals that the p65 subunit of NfκB stably integrates into the VDR transcription complex in TNF-α-treated bone cells and suggests that the elevation of TNF-α after menopause may contribute to acquired vitamin D resistance through this mechanism.

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