

## Annexin A4 interacts with the NF- $\kappa$ B p50 subunit and modulates NF- $\kappa$ B transcriptional activity in a Ca<sup>2+</sup>-dependent manner

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**Abstract** Previously, we identified annexin A4 (ANXA4) as a candidate substrate of caspase-3. Proteomic studies were performed to identify interacting proteins with a view to determining the roles of ANXA4. ANXA4 was found to interact with the p105. Subsequent studies revealed that ANXA4 interacts with NF- $\kappa$ B through the Rel homology domain of p50. Furthermore, the interaction is markedly increased by elevated Ca<sup>2+</sup> levels. NF- $\kappa$ B transcriptional activity assays demonstrated that ANXA4 suppresses NF- $\kappa$ B transcriptional activity in the resting state. Following treatment with TNF- $\alpha$  or PMA, ANXA4 also suppressed NF- $\kappa$ B transcriptional activity, which was upregulated significantly early after etoposide treatment. This difference may be due to the intracellular Ca<sup>2+</sup> level. Additionally, ANXA4 translocates to the nucleus together with p50, and imparts greater resistance to apoptotic

stimulation by etoposide. Our results collectively indicate that ANXA4 differentially modulates the NF- $\kappa$ B signaling pathway, depending on its interactions with p50 and the intracellular Ca<sup>2+</sup> ion level.

**Keywords** Annexin · Annexin A4 · Ca<sup>2+</sup> · Etoposide · NF- $\kappa$ B

### Introduction

Annexin A4 (ANXA4) is a member of the annexin (ANX) protein family, which contains common ANX repeat domains and binds phospholipids in a Ca<sup>2+</sup>-dependent manner. ANX proteins have diverse cellular functions, including vesicle trafficking, cell division, apoptosis, Ca<sup>2+</sup> signaling, growth regulation, and inflammation (see [1] for review). Moreover, these proteins are linked to various human diseases, such as cancer, cardiovascular disease, brain ischemia, and Alzheimer's disease [2–4]. Alterations in the expression levels of individual ANXs have been associated with tumorigenesis in several cancer types. ANXA4 was first identified as Ca<sup>2+</sup>- and lipid-binding porcine protein II [5], and subsequently shown to self-associate on membrane surfaces and aggregate phospholipid membranes [6]. ANXA4 expression is generally increased in renal, gastric, and colorectal cancer [3, 4, 7, 8]. In addition, the amount of ANXA4 was significantly increased in the hippocampi of postmortem brains of alcoholic patients, compared to controls [9] and in cultured cells following ethanol exposure [10]. The over-expression of ANXA4 in rat glioma C6 cells enhanced ethanol-induced cell lesions, accompanied by NF- $\kappa$ B activation [11]. However, the mechanisms underlying ANXA4 involvement in various

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diseases and alteration of physiological processes remain to be determined.

NF- $\kappa$ B, one of the most extensively characterized transcription factors in mammalian cells, regulates numerous genes involved in the immune response, cell proliferation, differentiation, survival, and apoptosis [12–16]. The NF- $\kappa$ B family is composed of five members, specifically, p65 (RelA), p105/p50 (NF- $\kappa$ B1), p100/p52 (NF- $\kappa$ B2), c-Rel, and RelB [15, 16]. These proteins contain the Rel homology domain (RHD), an N-terminal segment of approximately 300 residues responsible for DNA binding, dimerization, and nuclear translocation. The C-terminus contains a domain that interacts with inhibitors (I $\kappa$ -Bs). Recent reports have suggested that several additional proteins associated with Rel proteins determine the affinity and specificity of NF- $\kappa$ B [17–19]. The NF- $\kappa$ B activation signal is triggered by various stimuli, including cytokines, growth factors, death receptors, and DNA-damaging agents.

In a previous study, we identified ANXA4 as a candidate caspase-3 substrate using proteomic tools [20]. Here we performed interactome analysis to comprehensively determine the function of ANXA4, which revealed interactions with the p50 subunit of NF- $\kappa$ B. Our findings clearly indicate that ANXA4 differentially modulates the NF- $\kappa$ B signaling pathway via interactions with p50.

## Materials and methods

### Reagents, plasmids, and cell lines

Bosc 23, HEK-293, HeLa, and Caki-1 human kidney carcinoma were purchased from American Type Culture Collection (Rockville, MA). Media and other cell culture reagents were obtained from Gibco BRL (Grand Island, NE). The following antibodies were used in the study: mouse anti-FLAG and mouse anti-tubulin (Sigma, St. Louis, MO); mouse anti-Xpress (Invitrogen); rabbit anti-HA, goat anti-ANXA4, mouse anti-NF- $\kappa$ B p65, and goat anti-NF- $\kappa$ B p50 (Santa Cruz, CA); and mouse anti-I $\kappa$ -B $\alpha$  (Cell Signaling). To express ANXA4 in mammalian cells, we constructed a C-terminal FLAG-tagged human ANXA4 gene by PCR and cloned it into the pcDNA3.1/Zeo plasmid. The C-terminal HA-tagged p50 expression plasmids were generated by PCR and subcloned into pcDNA3.1/Zeo (Invitrogen). The Xpress/His-tagged p50 expression plasmids were generated by PCR and subcloned into pcDNA4/HisMax (Invitrogen). GST-tagged p50 was inserted into pEBG plasmid for protein expression. The cFLAG-ANXA4 D/E mutant expression plasmid was constructed by PCR using cFLAG-ANXA4 as a template according to a previous report [21].

### Immunoprecipitation and 2-DE

Bosc 23 cells were cultivated at 37°C in an atmosphere composed of 5% CO<sub>2</sub>. For transient overexpression, the expression vector was transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer's protocol. At 48 h after transfection, total protein lysates were incubated in lysis buffer (20 mM Tris, 137 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% NP-40) with protease inhibitors at 4°C overnight on a tube rotator with 40  $\mu$ l of either IgG control or anti-FLAG M2 agarose bead slurry (Sigma). Following three washes with lysis buffer, the bound protein was eluted from anti-FLAG beads with rehydration solution (9 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and, 2% ampholyte). The eluted solution was applied onto a pre-cast IPG strip with a linear pH range of 3–10. 2-DE was carried out using the Multiphor system for isoelectric focusing and the Protein II system for SDS-PAGE. After 2-DE, the proteins were visualized by silver staining as previously described [22, 23].

### Pull-down of His-tagged proteins

His-tagged p50 and cFLAG-tagged ANXA4 were co-transfected into Bosc 23 cells. Cells were lysed in Ni-NTA lysis buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 5 mM imidazole, and 0.05% Tween 20) and incubated at 4°C for 30 min. After centrifugation at 13,000 rpm for 30 min, cell lysates containing His-p50, and cFLAG-ANXA4 were mixed with Ni-NTA-agarose beads at 4°C for 6 h with rotation. Nonspecifically bound proteins were removed by washing with wash buffer. Bound proteins were eluted with 1 $\times$  SDS-PAGE sampling buffer containing 250 mM of imidazole and were then separated by 10% SDS-PAGE followed by immunoblotting with an anti-FLAG antibody.

### Luciferase reporter assay

Cells were routinely co-transfected with a TK-Renilla luciferase plasmid (Promega, Madison, WI) to normalize for transfection efficacy. The dual luciferase reporter assay kit from Promega was used following the manufacturer's protocol. Luciferase activity was measured with a luminometer Lumat LB9507 (EG&G Berthold). The values shown represent an average of three experiments in which each sample was performed in triplicate.

### RNA interference and transduction

To knock down ANXA4, the pSIREN-RetroQ-DsRed Express retroviral vector (Clontech) was employed. shRNA was designed by selecting a target sequence

specific for the human ANXA4 gene, as described previously (Sigma-Aldrich). The following gene-specific sequences were used to successfully inhibit ANXA4 expression: top, 5'-GAT CCG CAC ACT TCA AGA GAC TCT ATT CAA GAG ATA GAG TCT CTT GAA GTG TGC TTT TTT AAG CTT G-3'; and bottom, 5'-AAT TCA AGC TTA AAA AAG CAC ACT TCA AGA GAC TCT ATC TCT TGA ATA GAG TCT CTT GAA GTG TGC G-3'. These two oligonucleotides were annealed and sub-cloned, according to the manufacturer's guidelines, and a non-targeting control shRNA (scrambled control) was obtained from Sigma-Aldrich. Retroviruses were subsequently produced by transiently co-transfecting GP2-293 cells with a retroviral vector and VSV-G plasmid with Lipofectamine 2000 (Gibco-Invitrogen). At 48 h after transfection, media containing retroviruses were collected, filtered with 0.45- $\mu$ m filters, and used to infect cells in the presence of polybrene (8  $\mu$ g/ml). Infected cells were selected using FACSaria cell sorter (BD Bioscience) and were then maintained in growth medium [24, 25].

#### Confocal microscopy

HeLa cells were seeded onto glass cover slips and co-transfected with the indicated combinations of the expression plasmids. Transfectants treated with etoposide (final 20  $\mu$ M) for 4 h were then fixed/permeabilized with cytotoxic solution (BD Bioscience, Oxford) for 30 min at room temperature. After washing twice with PBS, fixed and permeabilized cells were incubated with Image-iTM FX signal enhancer (Molecular Probes) for 30 min. Incubation of cells with anti-FLAG and anti-HA in 1% BSA was carried out for 1 h at room temperature. After PBS washing, cells were subsequently incubated with Alexa Flour<sup>TM</sup> 546 anti-mouse IgG, Alexa Flour<sup>TM</sup> 488 anti-rabbit IgG, and Alexa Flour<sup>TM</sup> 660 anti-goat IgG (1:1,000 dilution; Molecular Probes) in 1% BSA for 1 h. Cell nuclei were stained with 406-diamino-2-phenylindole (DAPI). Finally, cells were mounted with a mounting solution containing 10% glycerol and then observed under a laser confocal microscope (Carl Zeiss).

#### Subcellular fractionation

The cells were washed with ice-cold PBS, left on ice for 10 min, and then resuspended in isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM DTT, 0.1 mM PMSF, and 10 mM Tris-HCl, pH 7.4) containing a protease inhibitor cocktail (Roche). After 100–300 strokes in a Dounce homogenizer, the nuclei fraction was fractionated at 80  $\times$  g for 10 min from the supernatant [23].

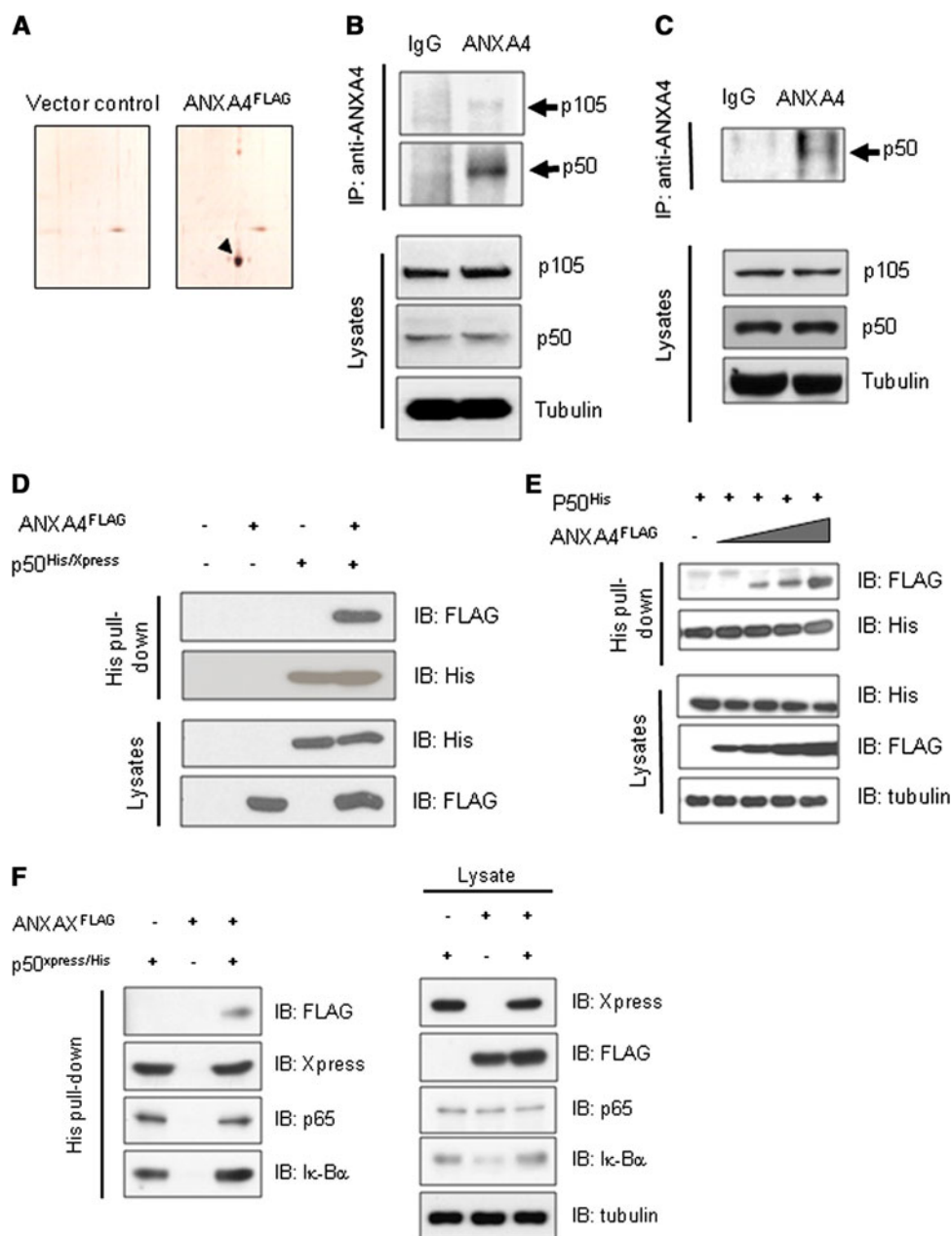
## Results

### ANXA4 interacts with the p50 subunit of NF- $\kappa$ B

Previously, we identified the ANXA4 protein as a candidate substrate of caspase-3 [20]. To further investigate the role of ANXA4 during apoptosis, we investigated proteins that interact with ANXA4 using proteomic tools. Extracts were prepared from Bosc 23 cells in which FLAG-tagged ANXA4 was expressed. FLAG-tagged ANXA4 was immunoprecipitated using an anti-FLAG antibody, and subjected to 2-DE analysis. Several spots appeared on the gel on which cell extracts expressing FLAG-tagged ANXA4 proteins were separated (Supplemental Fig. 1). These spots were identified by mass spectrometry. The protein list included NF- $\kappa$ B p105 (Fig. 1a and Supplemental Fig. 1), which activates genes involved in immune responses, proliferation, differentiation, and apoptosis. Consequently, we focused on characterizing the relationship between ANXA4 and the NF- $\kappa$ B signaling pathway. As a first step, *in vivo* interactions between endogenous ANXA4 and p105 were confirmed in Caki-1, a human renal carcinoma cell line that expresses high levels of ANXA4. As shown in Fig. 1b, endogenous ANXA4 interacts with not only p105 but also p50. The latter is the mature form derived from p105 through proteolytic cleavage. We did not find p50 spots in the 2-DE gel, possibly owing to its location near the heavy-chain band. Endogenous interactions between ANXA4 and p50 were also detected in HeLa cells (Fig. 1c). Moreover, interactions between ANXA4 and p50 were confirmed using His pull-down assays on extracts from cells overexpressing His-tagged p50 proteins and FLAG-tagged ANXA4 proteins, as shown in Fig. 1d. These interactions were enhanced with increased ectopic ANXA4 expression (Fig. 1e).

The p50 protein interacts with either p65 or p50 to form functional heterodimers or non-functional homodimers. The p50–p65 heterodimers are sequestered in the cytoplasm through interactions with I $\kappa$ -B in the resting state. Following application of various stimuli, these dimers are released from I $\kappa$ -B and translocate to the nucleus, where they activate the transcription of various downstream target genes [12–19]. To determine the effects of ANXA4 on the composition of the NF- $\kappa$ B complex, we performed immunoprecipitation experiments using extracts from control or ANXA4-overexpressing Bosc 23 cells. Our results clearly demonstrate that ANXA4 has no effect on the composition of the NF- $\kappa$ B complex (Fig. 1f).

Next, to determine the domain of p50 that interacts with ANXA4, we constructed four different kinds of deletion mutants tagged with GST, as shown in Fig. 2a. FLAG immunoprecipitation assays were then performed and were



**Fig. 1** ANXA4 interacts with p50 and p105, and has no effect on the composition of the NF- $\kappa$ B complex. **a** Enlarged image of a 2-DE gel showing the p105 spot in a control gel, compared to that in a gel presenting an ANXA4-transfectant. The detailed protocol is described in [Materials and methods](#). **b** Cell lysates were prepared from Caki-1 cells that overexpress ANXA4 protein. Extracts were immunoprecipitated with anti-ANXA4 goat polyclonal IgG or anti-goat preimmune IgG, separated by SDS-PAGE, and analyzed by Western blotting with NF- $\kappa$ B p105/p50-specific antibody for simultaneous detection of both proteins. **c** Endogenous interactions between ANXA4 and p50 were assessed in HeLa cells. Extracts were immunoprecipitated with anti-ANXA4 goat polyclonal IgG or anti-goat preimmune IgG, separated by SDS-PAGE, and analyzed by Western blotting with p50-specific antibody. **d** HEK-293 cells were transfected with vectors containing

FLAG-tagged ANXA4 and His/Xpress-tagged p50 genes, and extracted with Ni-NTA lysis buffer. Extracts were pulled down with Ni-NTA agarose beads and pellets analyzed by Western blotting. **e** Different amounts of FLAG-tagged ANXA4 DNA (0, 0.1, 0.5, 1, and 2  $\mu$ g) were transfected into HEK-293 cells with His-tagged p50 DNA (1  $\mu$ g). Transfected HEK-293 cells were extracted and pulled down using Ni-NTA agarose beads. Pellets were analyzed by Western blotting with anti-FLAG antibody. **f** FLAG-tagged ANXA4 DNA (1  $\mu$ g) was transfected into HEK-293 cells with His/Xpress-tagged p50 DNA (1  $\mu$ g). Transfected HEK-293 cells were extracted and pulled down using Ni-NTA agarose beads. Pellets were analyzed by Western blotting with anti-FLAG, anti-Xpress, p65, and I $\kappa$ -B $\alpha$  antibodies

followed by Western blotting analysis using an anti-GST antibody. All p50 mutants, except the RHD domain-deleted protein, interacted with ANXA4 (Fig. 2b), although these interactions were weaker than that of full-length p50 protein. These results strongly suggest that the entire RHD domain of p50 is required for interactions with ANXA4.

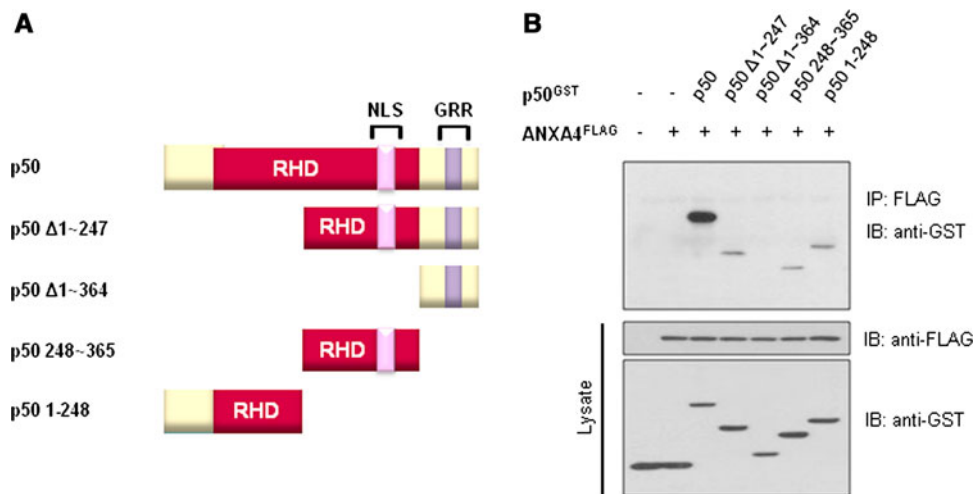
#### Transcriptional activation by NF- $\kappa$ B is reduced by ANXA4 overexpression

To explore the effects of ANXA4 on transcriptional activation of NF- $\kappa$ B, we performed luciferase assays using HeLa or HEK-293 cells overexpressing ANXA4. The ANXA4 protein contains a specific domain at the N-terminal end that differentiates it from other ANX proteins. Accordingly, we used C-terminal tagged ANXA4 in our transfection experiments. As shown in Fig. 3, ANXA4 reduced the transcriptional activity of NF- $\kappa$ B in a dose-dependent manner. Upon transfection of C-terminal FLAG-tagged ANXA4 into HeLa (Fig. 3a) or HEK-293 cells (Fig. 3b), the NF- $\kappa$ B signal pathway was fully suppressed in the resting state. Because the NF- $\kappa$ B signaling pathway is activated by various stimuli, we asked if ANXA4 reduces NF- $\kappa$ B transcriptional activity when the NF- $\kappa$ B signaling pathway is activated by these stimuli. In the initial experiment, we triggered the NF- $\kappa$ B signaling pathway with TNF- $\alpha$  in the presence or absence of ANXA4 overexpression and then measured luciferase activity over time (Fig. 3c). Overexpression of ANXA4 significantly reduced NF- $\kappa$ B transcriptional activity both before and after TNF- $\alpha$  stimulation. Similarly, overexpression of ANXA4 significantly decreased NF- $\kappa$ B transcriptional

activity in the presence of other stimuli, such as etoposide and phorbol 12-myristate 13-acetate (PMA) (Fig. 3d). These results indicate that ANXA4 disturbs the NF- $\kappa$ B signal pathway even when the NF- $\kappa$ B pathway is activated. In addition, it is notable that NF- $\kappa$ B activity was reliably upregulated in the early phase after treatment with etoposide (Fig. 3d). Genotoxic agents, such as etoposide, activate the NF- $\kappa$ B signaling pathway and upregulate the intracellular  $\text{Ca}^{2+}$  level [26]. Thus, the influence of ANXA4 on NF- $\kappa$ B signal activation at 4 h after etoposide treatment may be due to increased  $\text{Ca}^{2+}$  levels. The increased NF- $\kappa$ B activity by ANXA4 was disappeared at 12 h after etoposide treatment. Furthermore, the effect of ANXA4 on NF- $\kappa$ B activity after PMA treatment was reversed by the addition of ionomycin, which increases the intracellular  $\text{Ca}^{2+}$  levels (Fig. 3d). These results strongly imply that  $\text{Ca}^{2+}$  could be involved in ANXA4-mediated modulation of NF- $\kappa$ B transcriptional activity.

#### Transcriptional activity of NF- $\kappa$ B is upregulated upon knock-down of ANXA4 with RNAi

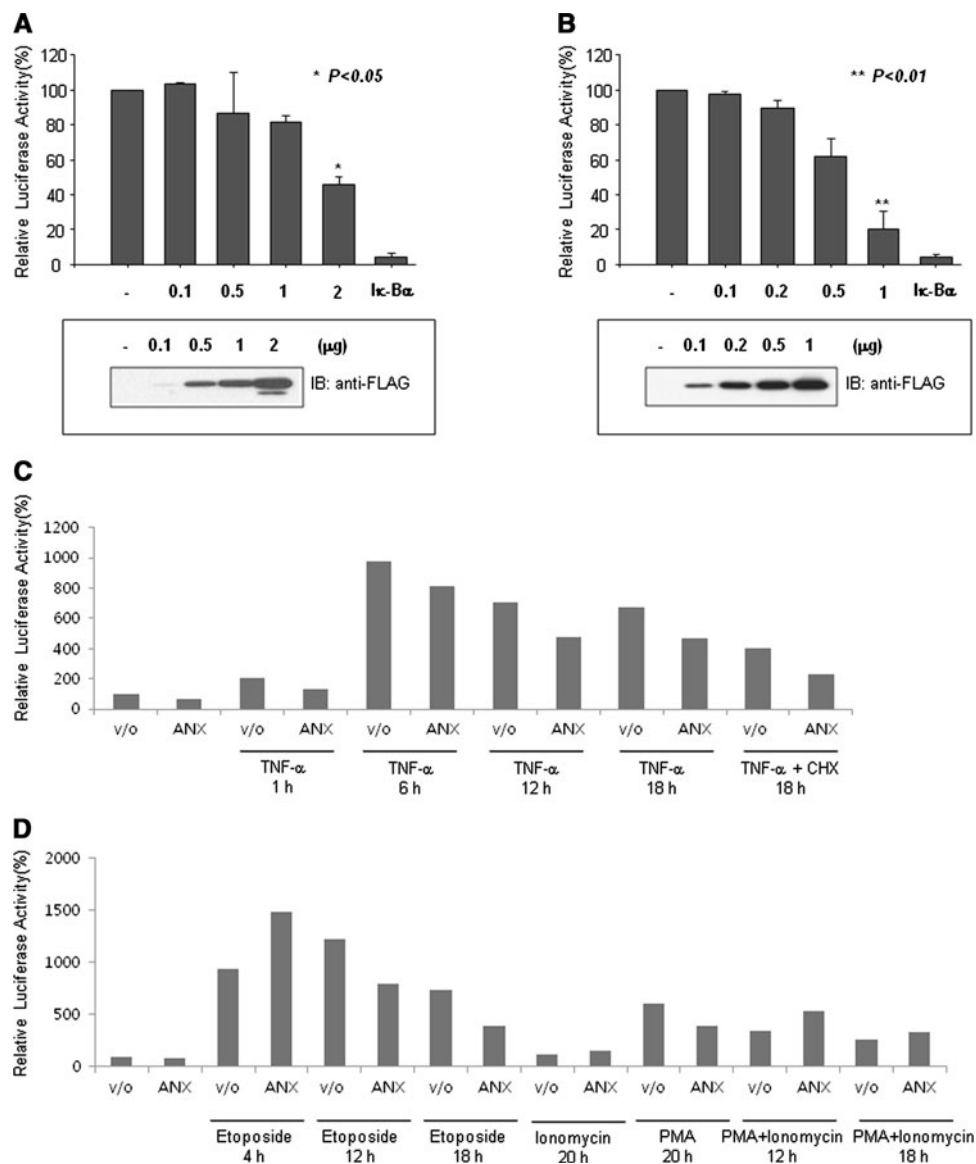
We attempted knock-down of ANXA4 with RNAi to confirm its role in NF- $\kappa$ B signaling. We infected HeLa cells with a retrovirus expressing ANXA4 shRNA or a scrambled control. Infected cells were isolated by FACS sorting and grown further. As shown in Fig. 4a, most cells were successfully infected with retrovirus expressing shRNA against ANXA4. Knock-down of endogenous ANXA4 expression was confirmed by Western-blot analysis (Fig. 4b). NF- $\kappa$ B reporter assays were then performed. As shown in Fig. 4c, knock-down of ANXA4 increased



**Fig. 2** p50 interacts with ANXA4 through its Rel homology domain. **a** Structural map of p50 and several deletion mutant proteins used in this study are depicted; the Rel homology domain (RHD), nuclear localization sequence (NLS), and glycine-rich region (GRR) are designated. **b** To determine the region of p50 required for association

with ANXA4, Bosc 23 cells were co-transfected with GST-tagged p50 truncation mutants and FLAG-tagged ANXA4. Transfected cells were subjected to GST pull-down assays, and analyzed by Western-blot analysis with the anti-FLAG antibody

**Fig. 3** NF- $\kappa$ B transcriptional activity assay after enforced expression of ANXA4. **a** The vector encoding C-terminal FLAG-tagged ANXA4 was transfected into HeLa cells at the indicated amounts and then luciferase assays were performed. Below the graph, the amounts of overexpressed ANXA4 proteins determined by Western blotting with an anti-FLAG antibody are shown. **b** The effect of C-terminal FLAG-tagged ANXA4 expression in HEK-293 cells on NF- $\kappa$ B transcription activity was investigated, as described in **a**. **c** The NF- $\kappa$ B transcriptional assay after TNF- $\alpha$  stimulation was performed in HeLa cells transfected with vector encoding C-terminal FLAG-tagged ANXA4. **d** The NF- $\kappa$ B transcriptional assay after etoposide or PMA stimulation was performed in HeLa cells transfected with a vector encoding C-terminal FLAG-tagged ANXA4. To ascertain whether Ca<sup>2+</sup> modulates NF- $\kappa$ B transcriptional activity induced by ANXA4, cells were co-treated with ionomycin and PMA

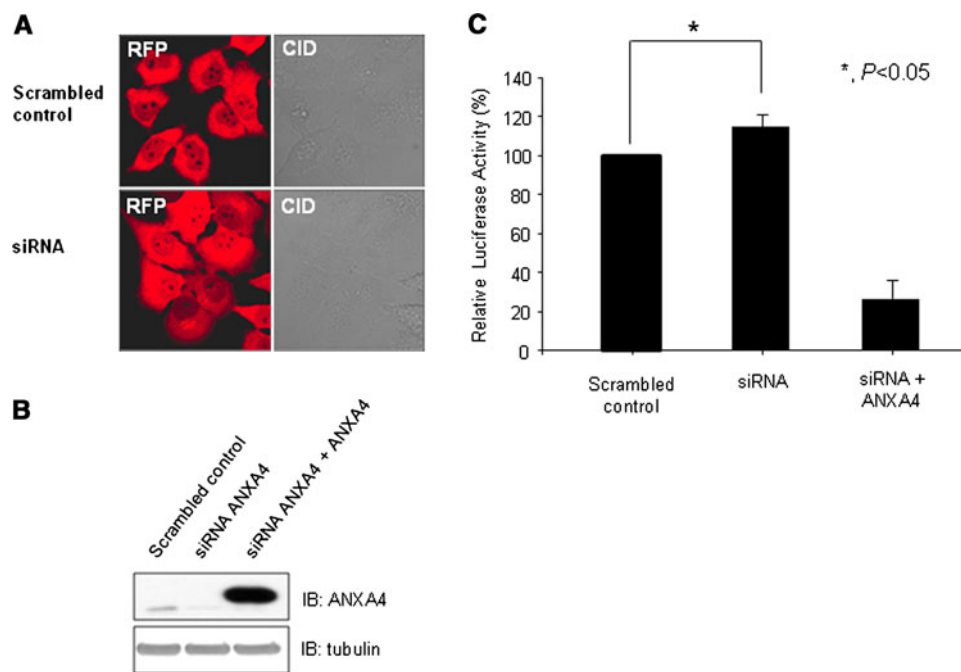


NF- $\kappa$ B activity compared to the scrambled control. We next infected ANXA4-KD HeLa cells with an ANXA4-overexpression retroviral vector. Overexpressed ANXA4 contains altered mRNA sequence in the region corresponding to the RNAi sequence (Arg-289, CGG[Arg]  $\rightarrow$  CCG[Arg]); thus, the expressed ANXA4 was not influenced by the RNAi system employed here (lane 3 in Fig. 4b). Figure 4c clearly shows that exogenous expression of ANXA4 led to a marked decrease in NF- $\kappa$ B transcriptional activity.

Ca<sup>2+</sup> induces enhanced interactions between ANXA4 and p50

ANXA4 is able to bind Ca<sup>2+</sup> through its conserved ANX domain. We next wondered whether Ca<sup>2+</sup> has an effect on

the interactions between the p50 protein and ANXA4. To examine this hypothesis, His pull-down assays were performed after changing the Ca<sup>2+</sup> level by exogenously adding CaCl<sub>2</sub>. As shown in Fig. 5a, Ca<sup>2+</sup> dramatically enhanced the interactions between p50 and ANXA4. In contrast, Mg<sup>2+</sup> exerted a marginal effect (Fig. 5b). To confirm in detail the effects of Ca<sup>2+</sup> on the interactions between these proteins, we constructed an ANXA4 D/E mutant that could not bind Ca<sup>2+</sup>. The ANXA4 D/E mutant still interacted with p50. In addition, Ca<sup>2+</sup> slightly promoted the interactions between the mutant protein and p50 (Fig. 5b). However, wild-type ANXA4 interacted with p50 several times more strongly than the D/E mutant. These results strongly suggest that the interactions between p50 and ANXA4 are enhanced by Ca<sup>2+</sup> through binding of Ca<sup>2+</sup> to the ANX domain of ANXA4.



**Fig. 4** NF- $\kappa$ B transcriptional activity assay after knock-down of ANXA4 with RNAi. **a** Monitoring of GFP expression using fluorescence microscopy after enrichment using the BD FACSARIA cell sorting system (BD Biosciences, MA). **b** Knock-down of endogenous ANXA4 expression was additionally confirmed by Western-blot analysis. Wild-type ANXA4 was overexpressed in

ANXA4-KD HeLa cells using a retrovirus system (pRetroX-IRES-ZsGreen1, Clontech, CA). The ANXA4 mRNA sequence corresponding to RNAi (Arg-289, CGG  $\rightarrow$  CCG) was mutated; thus, this enforced expressed ANXA4 was efficiently expressed in ANXA4-KD cells. **c** The NF- $\kappa$ B transcriptional assay was performed in ANXA4-KD cells using a reporter assay system

#### ANXA4 upregulates the transcriptional activity of NF- $\kappa$ B via increased $Ca^{2+}$ levels

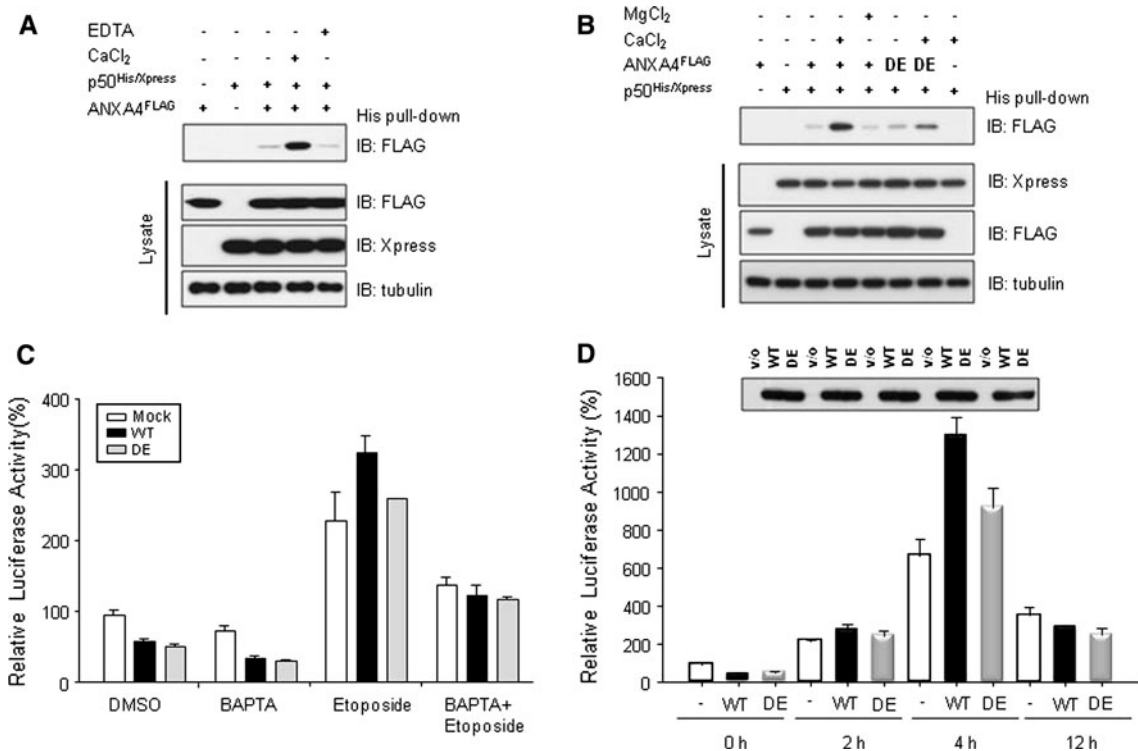
As specified above, interactions between ANXA4 and p50 were significantly enhanced by  $Ca^{2+}$ . We asked if  $Ca^{2+}$  has an effect on the NF- $\kappa$ B transcriptional activity induced by ANXA4. BAPTA-AM, a calcium chelator, was added together with etoposide to cells expressing vector only, ANXA4 or ANXA4 D/E mutant. As shown in Fig. 5c, BAPTA-AM blocked ANXA4-dependent NF- $\kappa$ B activation, but showed a marginal effect when the ANXA4 D/E mutant was overexpressed. Next, we performed luciferase assays in cells expressing wild-type ANXA4 or the D/E mutant over a time course after treatment with etoposide. As seen in Fig. 5d, overexpression of ANXA4 enhanced NF- $\kappa$ B activity approximately two-fold at 8 h after treatment with etoposide, while the ANXA4 D/E mutant enhanced NF- $\kappa$ B activity, but to a lesser extent, compared to wild-type ANXA4. By 12 h after treatment with etoposide, NF- $\kappa$ B activity in the presence of ANXA4 was no longer increased and in fact was below the level of the vector. These data indicate that overexpression of ANXA4 is able to enhance NF- $\kappa$ B activity triggered by genotoxic agents, such as etoposide, and is dependent upon  $Ca^{2+}$  ions.

#### Effect of ANXA4 on cell viability

To determine the role of ANXA4 in apoptosis, we assessed the expression level of *bax*, a typical downstream target gene of NF- $\kappa$ B, and performed cell viability assays after etoposide treatment. As expected, the *bax* level was significantly reduced after etoposide treatment in cells overexpressing ANXA4 (Fig. 6a). Furthermore, data from the cell viability assays clearly showed that overexpression of ANXA4 induced resistance against apoptotic stimulation by etoposide (Fig. 6b). In *cdk4*, one of the typical downstream targets of NF-AT, was not altered upon etoposide treatment (Fig. 6a), indicating that ANXA4 acts specifically on the NF- $\kappa$ B signal pathway.

#### ANXA4 co-translocates to the nucleus with p50 in the presence of high $Ca^{2+}$ level

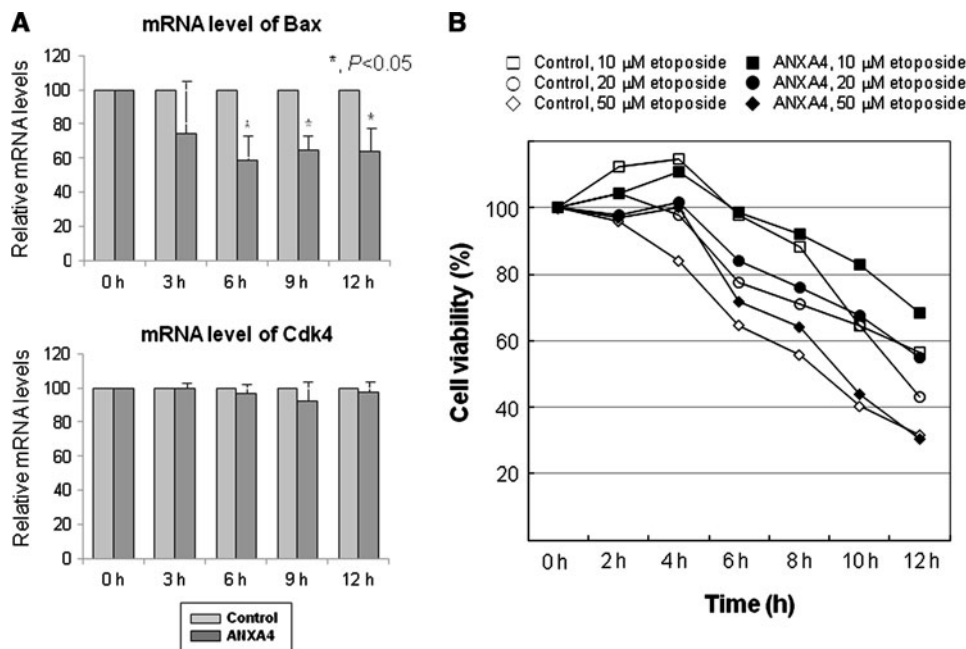
To determine whether ANXA4 translocates to the nucleus with the p50 subunit in response to etoposide, we performed confocal microscopic and subcellular fractionation analyses. As shown in Fig. 7a, the p50 subunit as well as the p65 subunit translocates to the nucleus after etoposide treatment. At that time, a portion of the ANXA4 in the cell seems to move to the nucleus together with the p50



**Fig. 5** ANXA4 interacts with p50 in a Ca<sup>2+</sup>-dependent manner. **a** To investigate the effects of Ca<sup>2+</sup> on interactions between ANXA4 and p50, cells transfected with His-tagged p50 and C-terminal FLAG-tagged ANXA4 were subjected to a His pull-down assay after treatment with CaCl<sub>2</sub> or EDTA, and analyzed by Western blotting with an anti-FLAG antibody. **b** To confirm the specificity of Ca<sup>2+</sup>, MgCl<sub>2</sub>, and the ANXA4 D/E mutant were used as negative controls for CaCl<sub>2</sub> and wild-type ANXA4, respectively. The ANXA4 D/E

mutant was constructed as described in Experimental Procedures. **c** BAPTA and the ANXA4 D/E mutant inhibited etoposide-induced NF-κB activation. **d** A time-course assay after treatment with etoposide. The transcriptional activity of NF-κB was reliably increased during early response to etoposide treatment and decreased significantly after 12 h of treatment. The effect of the ANXA4 mutant protein on enhanced transcriptional activity was not as significant as that of wild-type ANXA4

**Fig. 6** Overexpression of ANXA4 modulates the expression of *bax*, one of the NF-κB downstream target genes, and affects cell viability. **a** Real-time PCR analyses for assessing *bax* and *cdk4* mRNA levels of HeLa cells transfected with ANXA4 after apoptotic stimulus application (50 μM etoposide). **b** Cell viability assay of HeLa cells transfected with ANXA4 after etoposide treatment





subunit. The results from subcellular fractionation experiments also clearly show the translocation of ANXA4 into the nucleus after etoposide treatment (Fig. 7b). The ANXA4 D/E mutant did not seem to translocate to the nucleus, indicating that  $\text{Ca}^{2+}$  binding is critical for the translocation of ANXA4.

## Discussion

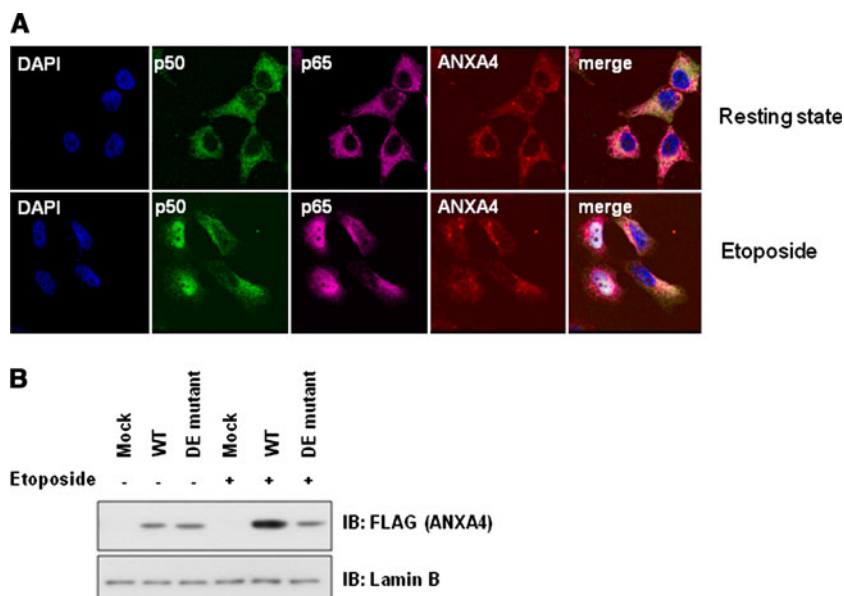
A number of physiological activities are attributed to ANXs, including regulation of membrane trafficking during exocytosis and endocytosis, mediation of cytoskeleton-membrane interactions, anti-inflammatory properties, and inhibition of phospholipase A2 [1]. In a previous study by our group, ANXA4 was identified as a caspase-3 substrate [20], supporting its involvement in the apoptotic pathway. Furthermore, many groups have reported that ANX proteins are linked to a number of human disorders, such as cancer, cardiovascular disease, brain ischemia, and Alzheimer's disease [2–4]. However, it is still unclear how ANXs are involved in these disorders. As the first step to elucidate the novel functions of ANXA4, several interacting proteins were identified using 2-DE/mass spectrometric analysis after immunoprecipitation. Among the candidate interacting proteins, p105, a component of the NF- $\kappa$ B complex was found. In both in vivo and in vitro analyses, we were able to detect the interaction of ANXA4 with p50, as well as p105, via the entire RHD domain of p50. We also found out that overexpression of ANXA4 did not affect interactions among p50, p65, and I $\kappa$ B. ANXA4 contains an ANX domain that interacts with  $\text{Ca}^{2+}$ , resulting in formation of a homo-trimer; this  $\text{Ca}^{2+}$ -binding ability suggests that  $\text{Ca}^{2+}$  might have an influence on the interaction between the

ANXA4 and p50 protein. Indeed, the addition of  $\text{CaCl}_2$  strengthened the binding between ANXA4 and p50, whereas EDTA weakened this interaction. In conjunction with data obtained using an ANXA4 mutant, these findings clearly demonstrate that the  $\text{Ca}^{2+}$  ion specifically enhances interactions between ANXA4 and p50.

NF- $\kappa$ B is one of the most extensively characterized transcription factors in mammalian cells, and it triggers the transcription of genes involved in inflammation, cell proliferation, differentiation, apoptosis, and survival, dependent on the receipt of proper upstream signals. It is therefore notable that ANXA4 influences NF- $\kappa$ B transcriptional activity. Based on the results obtained using a reporter assay system, we conclude that ANXA4 reduces transcriptional activation by NF- $\kappa$ B in a dose-dependent manner in the resting states (untreated cells). In addition, upon stimulation of cells with NF- $\kappa$ B activation signals, such as TNF- $\alpha$  or PMA, NF- $\kappa$ B transcriptional activity was suppressed by ANXA4. On the other hand, ANXA4 induced transcriptional activation of NF- $\kappa$ B early after treatment with etoposide. Why does ANXA4 respond differently to distinct types of NF- $\kappa$ B activation stimuli? This still needs to be determined. We propose that  $\text{Ca}^{2+}$  ions are the cause of the different responses between TNF- $\alpha$ /PMA and etoposide because etoposide, a genotoxic agent, uses  $\text{Ca}^{2+}$  ions as a signal transduction molecule, which is in contrast to TNF- $\alpha$  and PMA [27]. We also found that the interaction between p50 and ANXA4 became stronger when  $\text{Ca}^{2+}$  levels were elevated. These enhanced interactions induced translocation of ANXA4 into the nucleus with p50, consequently, leading to increased NF- $\kappa$ B transcriptional activity, and ultimately, enhanced cell viability against etoposide-induced death.

The well-known transcription factor NF- $\kappa$ B is affected by complex signaling networks within the cell. Here, we

**Fig. 7** ANXA4 translocates to the nucleus together with p50 after treatment with etoposide. **a** Changes in localization of p50, p65, and ANXA4 were analyzed using immunofluorescence microscopy after treatment with or without etoposide. Nuclei were stained with DAPI. **b** Subcellular fractionation analysis for detecting ANXA4 translocation after etoposide treatment. Lamin B was used as a nuclear marker protein



demonstrate that ANXA4 affects NF- $\kappa$ B signaling in a Ca<sup>2+</sup>-dependent manner. Previous studies have shown increased ANXA4 expression in renal clear cell carcinoma [7]. Additionally, reduced levels of ANXA4 are correlated with aggressiveness in prostate cancer [28]. Based on the experimental data, we speculate that ANXA4 may alter NF- $\kappa$ B signaling in various cancers. ANXA4 is also involved in the resistance phenotype against paclitaxel, an anti-cancer reagent, in human cancer cell lines [29]. Paclitaxel affects the cytosolic Ca<sup>2+</sup> signal by opening mitochondrial permeability transition pores [30]. In conjunction with earlier reports, the present results indicate that overexpression of ANXA4 imparts paclitaxel resistance to cancer cells by upregulating NF- $\kappa$ B activity in the presence of higher Ca<sup>2+</sup> levels. Until now, it has been unclear what the inter-relationship between apoptosis and ANXA4 is [31, 32]. However, based on results from this study, we suggest that ANXA4 participates in the apoptotic pathway by regulating NF- $\kappa$ B transcriptional activity. To understand the more detailed roles of ANXA4 in the apoptotic process, we are attempting to determine if ANXA4 is a real substrate of caspase-3.

Further studies are necessary to elucidate in detail the mechanisms by which ANXA4 modulates NF- $\kappa$ B-activating signals. At this time, we plan to investigate whether (1) ANXA4 is the only ANX family member that interacts with p50 (there are 12 ANX subfamilies [ANXA1–11 and ANXA13] in humans) and (2) ANXA4 directly interacts with other NF- $\kappa$ B component(s), such as p65 and I $\kappa$ -B.

To our knowledge, this is the first report to demonstrate that ANXA4 interacts with p50 and modulates NF- $\kappa$ B signaling in a Ca<sup>2+</sup>-dependent manner. The involvement of ANXA4 in NF- $\kappa$ B signaling provides a valuable clue towards understanding the mechanisms underlying diseases or altered cell signaling mediated by differentially expressed ANXA4.

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