Cyr61 Expression Confers Resistance to Apoptosis in Breast Cancer MCF-7 Cells by a Mechanism of NF-κB-dependent XIAP Up-Regulation*

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The aggressiveness of a tumor is partly attributed to its resistance to chemotherapeutic agent-induced apoptosis. Cysteine-rich 61 (Cyr61), from the CCN gene family, is a secreted and matrix-associated protein, which is involved in many cellular activities such as growth and differentiation. Here, we established a cell model system to examine whether stable expression of Cyr61 in MCF-7 cells can confer resistance to apoptosis and identify possible participating mechanisms. We showed that stable cell lines overexpressing Cyr61 had acquired a remarkable resistance to apoptosis induced by paclitaxel, adriamycin, and β-lapachone. Most interesting, gel shift and reporter assays showed that the Cyr61-overexpressing cells had significantly increased NF-κB activity compared with neo control cells. Blockage of NF-κB activity in Cyr61-expressing cells by transfecting with a dominant negative (DN)-IKK or with an NF-κB decoy rendered them more susceptible to anti-cancer drugs-induced apoptosis. In addition, several NF-κB-regulated anti-apoptotic genes were examined, and we found that only XIAP showed a significant 3–4-fold increase in mRNA and protein in Cyr61-overexpressing cells but not in neo control cells. Treatment with inhibitor of apoptosis protein (XIAP)-specific antisense, but not sense, oligonucleotides abolished the apoptosis resistance of the Cyr61-overexpressing cells. At the same time, transfection of these stable cells with DN-IκBα to block NF-κB activity also effectively reduced the elevated XIAP level. Function-neutralizing antibodies to α, β2, and β5 could inhibit Cyr61-mediated NF-κB activation as well as XIAP expression. Taken together, our data suggested that Cyr61 plays an important role in resistance to chemotherapeutic agent-induced apoptosis in human breast cancer MCF-7 cells by a mechanism involving the activation of the integrins/NF-κB/XIAP signaling pathway.

Apoptosis is a genetically controlled process that plays an essential role in embryogenesis, homeostasis, and the cellular response to stressful stimuli (1–3). Dys-regulation of apoptosis occurs commonly in a wide variety of human malignances (4, 5). The failure of cancer cells to undergo apoptosis induced by anti-neoplastic agents is a major problem in cancer therapy (6, 7). The aggressiveness of tumors is, in part, because of their acquired resistance to apoptosis (8, 9). Thus, unraveling the mechanisms of apoptosis in tumor cells could possibly facilitate effective therapeutic intervention against aggressive human cancers.

An emerging family of secreted, matrix-associated and immediate early genes that play diverse roles in angiogenic and growth regulation has been identified and named connective tissue growth factor (CCN2), Cyr61 (CCN1), and Nov (CCN3) proteins (10–13). This family of genes consists of six members with similar DNA sequences. By dissecting their protein structure, these CCN proteins are composed of four conserved modular domains that share sequence similarities with insulin-like growth factor-binding proteins, the von Willebrand factor type C repeat, the thrombospondin type 1 repeat, and the carboxy-terminal region containing cystine knot domains (14–16). It has become clear that the varied biological activities of the CCN proteins can possibly be attributed to a range of actions associated with their specific modular domains (13, 17–20).

Cyr61 (CCN1), one of CCN members, was originally identified by differential hybridization screening of a cDNA library of serum-stimulated BALB/c3T3 fibroblasts (21). Cyr61 is not expressed in quiescent fibroblasts but is rapidly activated by numerous growth factors such as epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β. Recently, Cyr61 was found to be up-regulated by 17β-estradiol in human breast cancer cells (11, 26, 27), suggesting that Cyr61 might have a role in mammary tumorigenesis. Overexpression of Cyr61 in MCF-12A normal breast cells induced tumor formation and vascularization in nude mice (27). Similarly, overexpression of Cyr61 in MCF-7 breast cancer cells induced estrogen independence and promoted the invasiveness of MCF-7 cells when transplanted into mice (26). Clinically, elevated levels of Cyr61

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††‡‡§§ The abbreviations used are: Cyr61, cysteine-rich 61; CCN, connective tissue growth factor; NF-κB, nuclear factor-κB; XIAP, inhibitor of apoptosis protein; RT, reverse transcriptase; mAb, monoclonal antibody; DN, dominant negative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline.
mRNA have been detected in primary breast tumors by real time RT-PCR assay (29). The level of expression of Cyr61 mRNA expression level is also positively correlated with more advanced features in breast cancer patients, such as tumor size and lymph node metastasis (29). Most interesting, Cyr61 overexpression has been shown recently (30) to suppress apoptosis induced by Taxol in MCF-7 cells. However, the detailed mechanism underlying Cyr61 protection from anti-cancer drug-induced death largely remains unknown.

In this study, we investigate whether overexpression of Cyr61 in breast cancer MCF-7 cells could modulate their sensitivity to apoptosis induced by various anti-cancer drugs. We found that NF-κB was constitutively activated in Cyr61-overexpressing breast cancer cells and was required for these cells to be resistant to anti-cancer drug-induced apoptosis. By utilizing RT-PCR and Western blot analysis, we found that XIAP, a NF-κB-dependent anti-apoptotic gene, was significantly up-regulated in Cyr61-overexpressing cells. Our results further delineate a novel mechanism for NF-κB-dependent up-regulation of XIAP that contributes to the anti-apoptotic activity of Cyr61.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The anti-cancer drugs Adriamycin, paclitaxel, and β-lapachone were kindly provided by Dr. Ruey-Long Hung, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan. Human anti-Cyr61 polyclonal antibody, anti-p65 monoclonal antibody, anti-β5 polyclonal antibody, anti-α2β1 polyclonal antibody, and anti-phosphotyrosine IκBα antibody were all purchased from Santa Cruz Biotechnology. Anti-αβ6 monoclonal antibodies were purchased from Neomarkers. α,β,δ-Poly(I):C (Poly(IC)) was obtained from Clontech, using TransFastTM (Promega). The primer sequences for cIAP-1 were 5'-AGAGCTTCGACCTTCTCGCCT-3' (F) and 5'-TCGAGGAGTTTCCCTCTCTCTGGCG-3' (R). The primer sequences for cIAP-2 were 5'-GAATATTCGAAAGGAGAGGAGATGACCGAGAAGACTG-3' (F) and 5'-TGGAGAAGAGGAGATGACCGAGAAGACTG-3' (R). The primer sequences for Bcl-xl were 5'-ACCACATCTGGGACCTGCTGGA-3' (F) and 5'-GGAGCTG- AAGGCTGGTTGAGGAGG-3' (R). The primer sequences for A20 were 5'-CACACAAGGCACATTGATCC-3' (F) and 5'-CAAGATTCATCTGGAGAAGG-3' (R). The primer sequences for XIAP were 5'-GGGCTATTGACCATCGACATCGACAGCAG-3' (F) and 5'-GGATACATGAGTCTGCAAACCG-3' (R). The primer sequences for α-actin were 5'-GATGATATATGCCTGGGCC-3' (F) and 5'-TGGGTCATCTTCTGGCGGT-3' (R). The reaction mixture was first denatured at 95°C for 10 min. The PCR conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25–35 cycles, followed by 72°C for 10 min. PCR products were purified by ethidium bromide staining after agarose gel electrophoresis.

**NF-κB/Rel-specific Decay Oligodeoxynucleotides and XIAP Oligonucleotide Treatment**—We used a phosphorothioate double-stranded decoy oligodeoxynucleotide (ODN) carrying the NF-κB/Rel-consensus sequence 5'-CTAAGGAGATTTCTTCCCC-3' / 5'-GGAACTGTCTCCAAAAGCAG-3'.

**Cell Cultures**—The human breast cancer cell line MCF-7 was kindly provided by Dr. Ruey-Long Hung. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum with 2 mM l-glutamine (InVitrogen), 100 μg/ml streptomycin, and 100 units/ml penicillin. Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

**Stable and Transient Transfections**—The expression vector Cyr61 was constructed by placing the human Cyr61 cDNA in the pcDNA3.1 eukaryotic expression vector containing the neomycin gene under the control of the same promoter. The dominant negative 32/36A mutated form of IκBα (DN-IκBα) was kindly provided by Dr. Shuang-En Chang. The constructs were transfected into MCF-7 cells by TransFastTM (Promega).

**RNA Isolation and RT-PCR**—Total RNA was isolated using RNeasy B according to the manufacturer's instructions. For reverse transcription, a 1-μg aliquot of total RNA was reverse-transcribed into single-stranded cDNA by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was amplified with the forward (F) and reverse (R) primers by PCR as described. The primer sequences for Cyr61 were 5'-CGAGGCTGAGGTGAGACCCACAC-3' (F) and 5'-AGGACTGCATAGCATTGACCTCTGCT-3' (R). The primer sequences for eIL-1α were 5'-CTGGAGAAGGAGATGACCGAGAAGACTG-3' (F) and 5'-TCGAGGAGTTTCCCTCTCTCTGGCG-3' (R). The primer sequences for cIAP-2 were 5'-GAATATTCGAAAGGAGAGGAGATGACCGAGAAGACTG-3' (F) and 5'-TGGAGAAGAGGAGATGACCGAGAAGACTG-3' (R). The primer sequences for XIAP were 5'-GGGCTATTGACCATCGACATCGACAGCAG-3' (F) and 5'-GGATACATGAGTCTGCAAACCG-3' (R). The primer sequences for α-actin were 5'-GATGATATATGCCTGGGCC-3' (F) and 5'-TGGGTCATCTTCTGGCGGT-3' (R). The reaction mixture was first denatured at 95°C for 10 min. The PCR conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25–35 cycles, followed by 72°C for 10 min. PCR products were purified by ethidium bromide staining after agarose gel electrophoresis.
and the colonies were counted and photographed. All experiments were carried out in triplicate.

Cell Viability Assay—The viability of the MCF-7 cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) as a substrate. The MTT assay is based on the activity of mitochondria dehydrogenases, which reduce the water-soluble tetrazolium salt to a purple insoluble formazan product. The amount of MTT formazan product was measured spectrophotometrically at 570 nm. Each individual experiment was repeated three times.

Apoptosis Analysis by Flow Cytometry—Trypsinized or pelleted cells were stained with 0.5 ml of 50 μg/ml propidium iodide and stained with 0.5 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) as a substrate. The MTT assay is based on the activity of mitochondria dehydrogenases, which reduce the water-soluble tetrazolium salt to a purple insoluble formazan product. The amount of MTT formazan product was measured spectrophotometrically at 570 nm. Each individual experiment was repeated three times.

DNA Condensation Detection of Fluorescence Microscopy—Cell grown on degreased glass coverslips to 60–80% confluence in regular culture medium were fixed in methanol/acidic acid (3:1, v/v) solution for 5–10 min and washed with PBS. The fixed cells were stained with 0.1 ng/ml Hoechst 33258 for 10 min in dark. The cells were observed and photographed under a Nikon fluorescence microscope.

Immunofluorescence—Cells grown on degreased glass coverslips to 60–80% confluence in regular culture medium were fixed in methanol/acidic acid (3:1, v/v) for 30 min at 4°C and permeabilized with 0.1% Triton X-100 in PBS for 5 min. These cells were then rinsed and blocked for 1 h in 5% fetal bovine serum at room temperature. The cells were then incubated with anti-p53 monoclonal antibody (Santa Cruz Biotechnology) or anti-p50 polyclonal antibody (Santa Cruz Biotechnology) and diluted 1:50 in PBS at 4°C overnight. After washing in PBS, the cells were incubated with a secondary fluorescein isothiocyanate-conjugated antibody (1:200, Sigma) for 1 h at room temperature. After extensive washing, the coverslips were inverted onto glass slides using Mowiol (Calbiochem) as a mounting medium. The slides were observed with a fluorescence microscope.

RESULTS

Expression of Cyr61 Confers Resistance to Apoptosis—To examine whether expression of Cyr61 would alter cellular sensitivity to apoptosis of breast cancer cells, the human breast cancer MCF-7 cell line, which exhibited an extremely low level of Cyr61 (27), was transfected with the human Cyr61 constitutive expression plasmid, pcDNA-3-Cyr61, and the control vector alone. After transfection, cells were cultured in a medium containing 300 μg/ml G418. Each colony that grew after G418 selection was picked and expanded. A mixed clone (Cyr61-M) was obtained by pooling together all of these single clones. Western blot analysis revealed that these were stable single clones, and the mixture expressed a 1.5–3.6-fold increase of Cyr61 protein compared with the vector control cells (Fig. 1A, upper panel). RT-PCR analysis showed that Cyr61 mRNA was also significantly elevated in these stable transfectants (Fig. 1A, lower panel). These Cyr61-overexpressed cells were subjected to further examination to determine their growth properties by using a trypan blue exclusion assay. The data demonstrated that the proliferation rate of these cells stably transfected with Cyr61 was very similar (Fig. 1B). Two representatives of the Cyr61-overexpressing cells (Cyr61-M and Cyr61 number 3) and neo control cells were analyzed for their

proteins were transferred to a nitrocellulose filter. The nitrocellulose filter was probed with the specific antibodies as indicated. Cyr61 mRNA was detected by RT-PCR, and β-actin acts as the internal loading control. B, Cyr61-M, Cyr61#3, Cyr61#8, and vector control cells (Neo) were plated at 1 × 10⁵ in 6-well plates. After culturing for different durations, the growth rates were measured by trypan blue exclusion assay. C, cells were seeded 1 × 10⁴ cells/well in soft agar plates as described under “Experimental Procedures.” After 2 weeks, the colonies were stained with crystal violet, photographed (upper panel), and counted (lower panel). Each experiment was performed in triplicate, and the results represent the mean ± S.D. of three experiments. Statistical significance was determined with a Student’s t test, and p values of <0.05 are indicated by asterisks.

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**Fig. 1.** Overexpression Cyr61 in MCF-7 cells increased anchorage-independent growth. A, determination of the protein (upper panel) and mRNA levels (lower panel) of Cyr61 in the Cyr61-transfected MCF-7 cells. The Cyr61-overexpressed clones (Cyr61-M, Cyr61#1, Cyr61#2, Cyr61#3, and Cyr61#9) and the vector control cells (Neo) were obtained as described under “Experimental Procedures.” Equal aliquots of protein extracted from these cells were electrophoresed, and the
CyR61 Inhibits Apoptosis via NF-κB/XIAP Pathway

After 24 h of starvation, Cyr61#3, Cyr61-M, and neo control cells were treated with β-lapachone (2.5 μM), adriamycin (2.5 μM), and paclitaxel (100 nm) for another 48 h. After treatment, the apoptotic cells were detected and quantified by Hoechst 33258 staining or flow cytometric analysis. Each experiment was performed in triplicate, and the results represent the mean ± S.D. of three experiments. Statistical significance was determined with a Student's t test, and p values of <0.05 are indicated by an asterisk. B, morphological examination of Cyr61-M and vector control (neo) cells after treatment with adriamycin (2.5 μM). The apoptotic characteristics, such as nuclear fragmentation and chromatin condensation (arrowheads), were determined by staining with Hoechst 33258 fluorescent dye. C, cells were treated with vehicle, paclitaxel (20 nm), or β-lapachone (2.5 μM) for 6 h, and replaced in fresh medium. After 2 weeks of culture, the colonies were stained with crystal violet, photographed (upper panel), and counted (lower panel). Each experiment was performed in triplicate, and the results represent the mean ± S.D. of three experiments. Statistical significance was determined with a Student's t test, and p values of <0.05 are indicated by asterisks.
Cyr61 Inhibits Apoptosis via NF-κB/XIAP Pathway

A.

![Graph showing expression levels of Neo, Cyr61-M, and Cyr61#3](image)

B.

![Graph showing anti-p65 and Hoechst staining](image)

**FIG. 3.** *Cyr61 expression activated the nuclear translocation of NF-κB.* A, nuclear extracts (50 µg) were prepared from MCF-7 cells stably expressing Cyr61 (Cyr61-M and Cyr61#3) and the control vector (Neo). Nuclear extracts were subjected to Western blot analysis by using the indicated antibodies for p65 or p50. Proliferating cell nuclear antigen was the positive control (PCNA). B, 4 × 10^5 cells were seeded on coverslips. After starvation for 24 h, immunostaining was performed by anti-p65 antibody followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. Nuclear localization of p65 was then observed by fluorescence microscopy (arrowheads). The position of the cell nucleus was confirmed by staining with Hoechst 33258 fluorescent dye.

Cyr61 is a novel ligand for integrins, and it is possible that signaling through integrin receptors may explain most of its diverse functions (13, 15, 19, 44–51). We thus tested whether integrin is involved in Cyr61-mediated NF-κB activation by using a luciferase reporter assay. To test this, we first transiently transfected Cyr61-M and neo control cells with the NF-κB reporter construct and then treated these cells with RGD peptides or function-blocking mAbs to the αvβ3 or αvβ5 integrins. As shown in Fig. 4C, RGD peptide treatment, but not control DGR peptide, significantly inhibited NF-κB activity in Cyr61-expressed cells. Again, function-blocking mAbs to integrin αvβ3 and αvβ5 also reduced greatly NF-κB activity (Fig. 4C). These results imply that Cyr61 activates NF-κB signaling mainly through an interaction with the αvβ3 and αvβ5 integrins.

**NF-κB Is Critical for Cyr61-mediated Anti-apoptosis**—We further explored whether NF-κB activation is involved in the Cyr61-mediated anti-apoptotic effect in breast cancer MCF-7 cells. To address this, Cyr61#3 cells were pretreated with 1 µM of NF-κB decoy oligonucleotide for 30 min, and this was followed by treatment with β-lapachone for a further 6 h. Fig. 5A shows that the NF-κB decoy oligonucleotide, but not the control scrambled oligonucleotide, enhanced β-lapachone-induced apoptotic cell death in Cyr61#3 cells. We further transfected DN-IκB vector into Cyr61#3 cells to examine their susceptibility to paclitaxel. Again, the reduction in NF-κB activation by transfection with DN-IκB greatly sensitized Cyr61-overexpressing cells to paclitaxel-elicted cell killing activity as demonstrated by the clonogenic assay (Fig. 5B). These experimental findings suggest that NF-κB activity is required for the Cyr61-mediated anti-cell death effect in MCF-7 cells.

**XIAP Acts as a Downstream Effector of Cyr61**—The question remains as to which gene or genes are the possible downstream effector genes that contribute to the Cyr61-mediated NF-κB-dependent cell survival effect. It has been known that several anti-apoptotic genes such as Bcl-xL, survivin, XIAP, cIAP, etc, are the transcriptional targets of NF-κB signaling (52–65). To answer this question, we analyzed the expression of these anti-apoptotic genes in Cyr61-expressing cells using RT-PCR. Fig. 6A reveals that of the genes analyzed only the mRNA of XIAP was substantially increased in Cyr61-overexpressed cells compared with neo control cells. Inhibition of NF-κB by transfection with DN-IκB significantly reduced the amount of XIAP mRNA in Cyr61#3 cells, suggesting the NF-κB pathway is required for the Cyr61-induced increase in XIAP mRNA (Fig. 6B). The level of XIAP mRNA was also greatly diminished by treatment with function-blocking mAbs to αvβ3 and αvβ5 integrins but was not affected by control IgG (Fig. 6B). Western blot analysis further confirmed that the increase in XIAP protein present in Cyr61#3 cells was also effectively attenuated by transfection with DN-IκB and by treatment with anti-αvβ3 or αvβ5 integrin mAbs (Fig. 6C).

To ascertain the role of XIAP in Cyr61-mediated cell survival effect, we treated Cyr61#3 cells with a XIAP-specific antisense oligonucleotide and then examined their sensitivity to apoptosis. Upon transfection with 5 µM of XIAP antisense oligonucleotide for 24 h, the protein level of XIAP was strongly decreased in Cyr61#3 cells. Control sense oligonucleotide did not affect the XIAP protein level (Fig. 7A). Transfection with control or antisense XIAP oligonucleotide marginally affected the level of XIAP in neo control cells (Fig. 7A). Under this condition, the paclitaxel- or β-lapachone-induced apoptosis was clearly prevented by the XIAP-specific antisense oligonucleotide but not by its control sense oligonucleotide (Fig. 7B). In addition, the antisense oligonucleotide treatment alone did not cause any signs of apoptosis in tested cells (data not shown). These results confirmed a role for XIAP in Cyr61-mediated anti-apoptotic activity.

**DISCUSSION**

Cyr61 exhibits a range of diverse functions that regulate different cellular activities. For cell survival activity, Cyr61 has been shown to augment activity in endothelial cells by an unknown mechanism, and this might account in part for its angiogenic effect (13, 45, 66). Recently, Menendez et al. (30) have demonstrated that Cyr61 expression in MCF-7 cells confers resistance to paclitaxel-induced cell death. Of interest, their preliminary data indicated that paclitaxel-induced p53 expression was reduced in Cyr61-overexpressed cells, suggesting that Cyr61 may interfere with p53 function. In addition, they further pointed out that the survival pathway PI3K/Akt was activated in Cyr61-overexpressed MCF-7 cells. However, the detailed mechanism by which Cyr61 protect cells from apoptosis induced by chemotherapeutic agents has not been characterized. In agreement with other studies, we have shown here that overexpression of Cyr61 significantly increases the resistance of MCF-7 cells to doxorubicin, paclitaxel, and β-lapachone but not to topotecan and etoposide (data not shown).
This indicates that Cyr61 displays a broad range of activity against chemotherapeutic agents, and it appears to activate a primary mechanism to protect cells from death. Under such a scenario, we have dissected, for the first time, the molecular mechanism by which Cyr61-conferred cell survival activity is mediated by NF-κB-dependent XIAP up-regulation.

The role of NF-κB in drug resistance has been extensively exploited in different cell systems (8, 33, 54, 68–78). Most important, NF-κB activity is elevated in many human breast tumors (31, 33, 37), and its activation conferred resistance to chemotherapeutic agents in MCF-7 cells (30). Here we provide evidence that the p65 and p50 NF-κB subunits are predominantly localized in the nucleus of the Cyr61-expressed cells (Fig. 3, A and B). The DNA binding activity and NF-κB pro-
Neo and Cyr61#3 cells were transfected with 1 μM/H9262. Inhibition of NF-κB was observed in a variety of different breast cancer cells, and inhibition of the NF-κB pathway has occurred in Cyr61-overexpressing cells. When Cyr61-overexpressing cells were treated with DN-IκB or NF-κB decoy, they became susceptible to apoptosis induced by anticancer drugs. Thus, our data suggest that the NF-κB signaling pathway is crucial to Cyr61-induced anti-apoptotic activity. Supportive of our current findings, several studies have demonstrated that constitutive activation of NF-κB is a frequent occurrence in a variety of cancers, including Hodgkin’s lymphoma, melanomas, and breast tumors (8, 31, 33, 37, 68–80).

Constitutive NF-κB activation has also been observed in a variety of different breast cancer cells, and inhibition of NF-κB activity has been shown to lead to apoptosis (33). Heregulin or Her-2Neu expression has been found recently to enhance breast cancer cell resistance to apoptosis through activation of NF-κB (81, 82). Our previous studies (42) have shown the activation of NF-κB in MCF-7 cells by heregulin is mediated by activation of p38 kinase. However, we have failed to detect the activation of the p38 signaling pathway in Cyr61-overexpressing MCF-7 cells (data not shown), suggesting that p38 kinase pathway is not involved in Cyr61-induced NF-κB activation. Instead we have detected the activation of the NF-κB signaling pathway is crucial to Cyr61-induced anti-apoptotic activity.
with our findings, Menendez et al. (30) have also shown that the PI3K pathway is activated in Cyr61-expressing cells, and inhibition of this pathway by the specific inhibitor wortmannin caused the cells to undergo apoptosis. Collectively, it appears that the PI3K/Akt/NF-κB signaling pathway is required for the anti-apoptotic effect of Cyr61.

XIAP, one of the members of the IAP family, is well established as an inhibitor of various different kinds of caspases (83–85). Through inhibition of caspase activity, XIAP can prevent apoptosis in a variety of cell systems in response to cytotoxic stresses, including anti-cancer drugs (83, 84, 86, 87). Although the detailed mechanism of how XIAP modulates caspase activity has yet to be determined, the role of XIAP in human cancer drug resistance is of great interest. This issue is further strengthened in our current study where antisense XIAP treatment not only reduced Cyr61-induced elevated XIAP mRNA and protein but also significantly enhanced the drug sensitivity of Cyr61-expressing cells. Recently, XIAP has been shown to be elevated in many human breast cancer cell lines and tumor specimens but not in normal cells or tissues (86). Inactivation of XIAP in human breast cancer cells causes the apoptosis of these cells (86). The functional blockage of XIAP by using synthetic Smac/DIABLO peptides also enhances the efficacy of chemotherapeutic agents in human breast cancer cells (28). Thus, our study and studies by others have provided evidence that XIAP may be a critical factor in modulating apoptosis in human breast cancer cells, and its expression can be regulated by Cyr61. Except for XIAP, we did not find any other NF-κB-regulated IAP gene family that was up-regulated in Cyr61-expressing MCF-7 cells. This specific linkage between XIAP and Cyr61 expression in breast cancer cells is particularly intriguing. Accumulating evidence shows that XIAP can stimulate NF-κB activation by increasing nuclear translocation of the p65 subunit (28, 67). This implies a positive loop between XIAP and NF-κB, and this may function coordinately to protect cell from apoptosis triggered by chemotherapeutic agents. Cyr61 seems to play a critical role in activating this protective loop.

The Cyr61 protein has been shown to exert a range of diverse functions, although not all, by binding with cell surface integrins including αvβ3, αvβ6, αvβ1α6β1, and αvβ1 (19, 45, 50). A fascinating finding made by Menendez et al. (30) showed that functional blocking of the αvβ3 integrin receptor in Cyr61-expressing MCF-7 cells led to sensitization in taxol-induced apoptosis, and this suggests that the αvβ3 integrin is involved in the Cyr61-induced signaling pathway and the cell survival effect. They also have proposed that focal adhesion kinase, a tyrosine kinase, which has a functional interaction with the integrins and the PI3K/Akt pathways, is located downstream of the αvβ3 integrin/Cyr61 signaling. Consistent with this notion, this work presents one more piece of evidence that strengthens this central theme by showing that Cyr61 overexpression in MCF-7 cells indeed activates a novel mechanism by which Cyr61-induced NF-κB activation as well as XIAP up-regulation appear to be dependent on the αvβ3/αvβ6 integrins.

In conclusion, stable overexpression of Cyr61 increased MCF-7 cells resistance to some anti-cancer drug-induced apoptosis. In stable cells, increased Cyr61 expression is correlated with increased activation of NF-κB and its downstream gene XIAP. Blockage of NF-κB activation led to a decrease in the XIAP levels, and this, in turn, sensitizes the cells to apoptosis. The Cyr61-mediated NF-κB activation and the resultant XIAP increments are effectively attenuated by inactivating the functioning of the integrins αvβ3 and αvβ6. In Fig. 8, we propose a detailed mechanism describing the underlying role of Cyr61 in chemotherapy agent-induced apoptosis via an integrins/ NF-κB-dependent up-regulation of XIAP.

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Cyr61 Expression Confers Resistance to Apoptosis in Breast Cancer MCF-7 Cells by a Mechanism of NF-κB-dependent XIAP Up-Regulation

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