

Co-relation with novel phosphorylation sites of I κ B α and necroptosis in breast cancer cells

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Abstract

Background: The phosphorylation of NF-kappaB inhibitor alpha (I κ B α) protein is pivotal to the regulation of NF- κ B transcription factor activity in the cell. The phosphorylation of I κ B α by I κ B kinase family have been so many identified, but the phosphorylation sites of I κ B α by other kinases remain poorly understood. We investigated the new phosphorylation site for I κ B α and identified its biological function in breast cancer cells.

Methods: Previously we observed that aurora kinase (AURK) binds I κ B α in the cell. To identify the essential domains of I κ B α for phosphorylation of I κ B α by AURK, kinase assay was performed with a series of I κ B α truncation mutants. AURK significantly promotes activation of I κ B α at serine 32, but not serine 36 residues, unlike I κ B kinase (IKK) family proteins activate both I κ B α serine residues. We also confirmed phosphorylation of I κ B α by the matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and nano-liquid chromatography hybrid quadrupole-Orbitrap mass spectrometer (nanoLC-MS/MS; Q Exactive).

Results: We identified two novel sites of serine phosphorylation at S63 and S262. Alanine transition of S63 and S262 (S63A and S262A) of I κ B α induced inhibition of cell proliferation and suppression of p65 transcription activity. Besides, S63A and/or S262A of I κ B α regulated apoptotic and necroptotic effects in breast cancer cells.

Conclusions: Therefore, we identified novel phosphorylation site of I κ B α by AURK, and its site was related to apoptosis and necroptosis pathway in breast cancer cells.

Background

Breast cancer is the most frequent malignant tumor in women and the leading cause of cancer death, since 30% of breast cancers develop distant metastases after the initial treatment of the apparently localized tumors[1]. Nowadays, the mechanisms underlying the genesis and progression of breast cancer are better understood[2, 3], but despite an improvement of the survival rates for variable protein interaction of breast cancer, we still have to go a long way to know the cure for all patients [2, 4].

Protein-protein interactions (PPI) mean that two or more proteins bind each other, to perform their biological functions[5]. Most of the important molecular processes in the cell, such as DNA replication, are performed by large molecular network of protein-protein interactions.[6] Those are built of a large number of protein elements organized by their PPI [5, 6]. Also, Interactions of proteins are important for many biological functions[7]. For example, external signals are transmitted to the interior of the cell by PPI[6, 7]. This process, called signal transduction, plays the essential role in many biological processes and diseases, such as cancer. Protein-protein interactions are the primary mechanism for virtually every process in a living cell[7]. We need to acquire more knowledge of various protein-protein interactions in order to understand biological phenomena, including diseases, and to provide the basis for new therapeutic approaches.

I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) is a member of a family of cellular proteins to inhibit the NF- κ B transcription factor[8]. I κ B α inhibits the NF- κ B by keeping them in a state isolated from the inactive cytoplasmic mask and the nuclear localization signal of NF- κ B proteins.[8] Furthermore, I κ B α blocks the ability of NF- κ B transcription factors binding to DNA, which is required for proper function of NF- κ B.[9] However, NF- κ B activation signal induce the activation of a putative protein-tyrosine phosphatase(s), leading to I kappa B-alpha serine 32/36 phosphorylation and degradation and NF- κ B nuclear translocation[10]. Some Hodgkin lymphoma cells have the mutated gene part of encoding the protein I κ B α [11]. These mutations inactivate the I κ B α protein[11]. Thus, NF- κ B to become active on the lymphoma tumor cells chronically, this activity contributes to the malignant state of these tumor cells[8, 9, 12]. On the other hand, Inhibition of NF-kappaB/Rel induces apoptosis of variable normal or cancer cells [13].

AURK protein is one member of the Aurora subfamily of serine/threonine protein kinases that are essential for cell proliferation.[14] Aurora kinase subfamily comprise three varieties; Aurora-A, Aurora-B and Aurora-C. AURKA and AURKB are involved in mitosis (cell division producing identical daughter cells), and AURKC is involved in meiosis (sexual reproduction).[14, 15] This proteins is over-expressed in various cancer cell lines such as breast cancer,[16, 17] suggesting an involvement in oncogenic signal transduction.[17]

In the study reported here, MALDI-TOF MS and nanoLC-quadrupole orbitrap MS/MS were used to obtain sequence data on I κ B α phosphorylated *in vitro* by AURK, and identification of biological process by novel phosphorylation site.

Methods

Proteins Active proteins (IKK α , IKK β and Aurora kinase C; Signalchem, Vanier Place Richmond, BC, Canada), kinase buffer I and ATP stock solution were purchased from signalchem. Halo-Tag plasmidvector (pFN18A), Restriction enzyme blend (Sgf + Pme), KRX expression cell, L-rhamnose monohydrate and sequencing-grade modified trypsin were purchased from Promega (Madison, WI). Quikchange lightning site-directed mutagenesis kit was purchased from Agilent technologies. D- glucose and phosphoric acid (PA) were purchased from Sigma-Aldrich. 2,5-Dihydroxybenzoic acid (2,5-DHB) was purchased from Bruker Daltonics. Acetonitrile (ACN, ultrapure ACS reagent grade and methyl alcohol (high purity HPLC reagent) were purchased from USB corporation and samchun pure chemical. All other chemicals used were of ACS or HPLC grade. Primary antibody (anti-I κ B α monoclonal antibody) and anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology and Cell signaling Technology. Primary antibody (anti-phospho-I κ B α serine32; Cell Signaling Technology, CA, USA, serine63 and serine262 monoclonal antibody; Custom made antibody) and anti-rabbit secondary antibody were purchased from abcam and Cell signaling Technology. Primer and DNA sequencing data were purchased cosmo genetech (Seoul, Korea).

Cloning and mutagenesis of IκBα IκBα constructs were cloned into the Halo-Tag vector pFN18A (Promega) for E.coli overexpression between the restriction sites Sgf and Pme . Mutagenesis reactions of the IκBα single and double point mutant were performed with the Quikchange lightning site-directed mutagenesis kit (Agilent technologies). The following oligonucleotides were used as sense primers: S32A, 5'-CACGACGCCGGCCTG-3'; S36A, 5'-CTGGACGCCATGAAA-3'; S32/36A, 5'-CACGACGCCGGCCTGGACGCCATGAA-3'. The C-terminal deletion mutants were by insertion of new GAAGCAGCAGCTCACCGAGTGAGTTTAAACGAATTCTGGGCT-3'; CGCACCTCCACTCCATCTGAGTTTAAACGAATTCTGGGCT-3'; IκBα stop codon with following PCR antisense primers: IκBα 1-72, 5'-1-175, 1-277, CTGACACTAGAAAACCTTTGAGTTTAAACGAATTCTGGGCT-3'. The construction of the ankyrin repeat domain deleted IκBα 17 was used overlap PCR method with following PCR antisense and sense primers: IκBα 1-72/278-317, 5'-GAAGCAGCAGCTCACCGAGCAGATGCTGCCAGAGAGTG-3', 5'-GAAGCAGCAGCTCACCGAGCAGATGCTGCCAGAGAGTG-3'. The mutated PCR fragments also were cloned into E.coli overexpression vector pFN18A (Promega).

Overexpression of IκBα in KRX cell Full-length human IκBα was cloned into a pFN18A vector (Promega). The plasmid is transformed into KRX cell (Promega) grown in LB media (add to ampicillin) supplemented with glucose and rhamnose to induce expression without isopropyl-β-D- thiogalactoside (IPTG). After growing the cells to OD 600 of 0.5-0.6 at 37 °C, the temperature was reduced to 20 °C for overnight expression.

Purification of IκBα For IκBα purification, Cell pellets were resuspended in 5 × of HaloTag purification buffer (50 mM HEPES (Sigma-aldrich), pH 7.5 and 150 mM NaCl) supplemented with 1x Protease Inhibitor (Thermo scientific), and sonicated on ice using a vibra cell sonicator (5 min total time on; 1 min on / 1 min off; amplitude = 40-60, pulse = 4). Lysates were centrifuged at 10,000 ×g for 30 min at 4 °C and the supernatants were directly applied onto pre-equilibrated HaloLink resin (Promega) following manufacturer recommendations. Binding to the resin was conducted at room temperature for 1 hr with constant end-over-end rotation, followed by three washes, each for 5 min with 10 × purification buffer. Target proteins were released from the resin by proteolytic cleavage using a ratio of HaloTEV to settled resin in HaloTag purification buffer for 1 hr at room temperature. The supernatants containing the released protein of interest and TEV protease were carefully removed into new tube. For TEV protease removal, add of HisLink resin into the tube. Binding to the resin was conducted at room temperature for 20 min with constant end-over-end rotation. Spin at 1,000 ×g for 5 min, and transfer supernatant to another tube. protein quantification was conducted by the Bradford method.^[10] Bovine serum albumin was used for the calibration of sample protein quantity.

Cell culture. MCF-7 (KCLB60104, Korean Cell line Bank) and MDA-MB 231 (KCLB88064, Korean Cell line Bank) cells were cultured at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA), 4.5 g/L glucose, L-glutamine, and 1% penicillin/streptomycin.

Staining or Western Blot analysis For in-gel trypsin digestion, the gels were stained by Coomassie brilliant Blue-R250 (CBB, 3M co.). For deletion mutant of I κ B α size visualization, the gels were stained by silver staining (GE Healthcare, uppsala, Sweden). For Western blotting, the protein separated by electrophoresis were transferred to 0.2 μ Polyvinylidene fluoride (PVDF; Bio-Rad Laboratories Inc.) membranes in Transfer buffer (25 mM Tris, 192mM Glycine, 20% (v/v) methanol) at 48 mA for 12 hr using Mini Trans-Blot Cell system (Bio-Rad Laboratories Inc.). in order to confirm the protein expression level as aging is processing, the transferred membrane was incubated in 5% (W/V) skim milk with 50 mM Tris-buffered saline with Tween 20 (TBST, Intron biotechnology, 24.7 mM Tris, pH 7.8, 2.7 mM KCl, 137 mM NaCl and 0.05% Tween-20) for blocking, and exposed to primary antibody (RIP3, MLKL, Caspase-3, Caspase-8, beta-actin: Cell Signaling Technology, CA, USA) overnight at 4 °C. After washing step (three times for 15 min with fresh TBST), the membrane was exposed to secondary antibody for 2 hr at room temperature. After washing step, the proteins were visualized using ECL solution and detected by ChemiDoc XRS+ system (Bio-Rad Laboratories Inc.). The transcription activity of p65 was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Mineapolis, MN, USA), performed according to the manufacturer's instructions. In the NF- κ B -p65 assay, a double stranded DNA sequence containing the NF- κ B response element was immobilized onto the bottom of the wells of a 96-well plate. NF- κ B contained in a nuclear extract binds to the NF- κ B response element, and then it was detected using an anti NF- κ B p65 antibody. A secondary antibody conjugated to HRP was added to provide a colorimetric readout at 450 nm.

In vitro kinase assay Recombinant active AURK (100 ng) (Signalchem, BC, Canada) was incubated with 10 μ M ATP (Signalchem, BC, Canada), 20 μ l kinase buffer (25 mM HEPES, 25 mM β - glycerophosphate, 25 mM MgCl₂, 2mM dithiotreitol, and 0.1 mM NaVO₃) and purified inactivated I κ B α . Reactions were incubated at 37°C for 30 min and terminated by addition of Laemmli SDS sample dilution buffer (Bio-rad, CA, USA). Proteins were separated by SDS-PAGE, and phosphorylation was detected specific antibody.

Cell Growth Assay. Cell Growth Assay. Breast cancer cells growth rates were measured using the WST-1 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Sigma-Aldrich, CA, USA) method. First, the cells were seeded in a 96-well plate at 5 x 10³ cells/well, incubated at 37°C for 24 hours, transferred to serum-free medium, and transfected with S63A and S262A as described above. Then, after cultivation under 21% O₂ for 24 hours, the cells were transferred to a culture medium containing 10% FBS. Finally, WST-1 was added to the wells, and the plates were incubated at 37°C for 3~4 hours for efficient cell dyeing, and analyzed for its absorbance at 460 nm using a spectrophotometer (Molecular Devices, USA). And, cell viability was measured by trypan blue staining. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. When a cell suspension is simply mixed with the dye, add equal parts of 0.4% trypan blue dye to the cell suspension to obtain a 1 to 2 dilution, and incubate mixture for less than three minutes at room temperature. Place the hemacytometer with 1:1 trypan blue staining cells on the stage of a light microscope (Olympus Optical Co., Tokyo, Japan) and calculated the staining cells.

Cell death assay-FACs Cells were stained with FITC-labeled annexin V and propidium iodide, and tumor cell death was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Millipore, Billerica, MA, USA) assay and flow cytometry (BD- Flow JO).

In gel trypsin digestion The in-gel digestion of phosphorylated I κ B α was excised from the polyacrylamide gel, washed with water and 30% methanol, and destained with 50% acetonitrile/10 mM ammonium bicarbonate (ABC) at room temperature until the CBB color was removed. The gel pieces were dehydrated with 100% (v/v) acetonitrile. After drying the organic solvent using Speed vacuum concentrator. Disulfide bonds were reduced with DTT (10 mM, 56 °C, 1 hr), and the free sulfhydryl groups were alkylated with iodoacetamide (55 mM, room temperature, 40 minutes in the dark). The gels were vortexed and completely dried in Speed vacuum concentrator. Gel pieces were washed with water, and dehydrated with 100% acetonitrile. After drying with a Speed vacuum concentrator, the gel was rehydrated using 100 ng/ μ l trypsin (50 mM ABC, pH 8.3) and the digestion was carried out at 37 °C for the maximum 20 hr for full digestion. The tryptic peptides were collected in extraction steps using 50% (v/v) acetonitrile containing 0.1% (v/v) formic acid. The tryptic peptide extracts were pooled and dried in Speed vacuum concentrator. The peptides were redissolved in 1% phosphoric acid for mass spectrometric analysis.

Mass spectrometry Phosphorylation of I κ B α were analyzed by both matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and nanospray-liquid chromatography hybrid quadrupole-Orbitrap mass spectrometer (nanoLC-Q Exactive Plus). For MALDI MS, 0.5 μ l of sample and 0.5 μ l of matrix solution were mixed followed by sample mixture was spotted onto a stainless-steel MALDI plate. The matrix solution was prepared by supernatant from a saturated solution of 2,5-dihydroxybenzoic acid (2,5-DHB) with 50% acetonitrile/1% phosphoric acid.^[12] The spots were analyzed with the ultrafleXtreme™ MALDI TOF-TOF mass spectrometer (Bruker Daltonics). MS spectra were collected in reflectron mode over the set mass range of $m/z = 700-3500$. Instruments were calibrated using Bruker peptide standard II. For LC-MS/MS, tryptic peptides were loaded on a trapping column with 75 μ m inner diameter, packed with 5 μ m C18 particles (Acclaim PepMap100, Thermo Scientific) and analyzed using a 15 cm analytical column packed with 2 μ m C18 particles (Acclaim PepMap RSLC, Thermo Scientific). Reversed phase chromatography was performed using an Ultimate 3000 RSLC nano system (Thermo Scientific) with a binary solvent consisting of 0.1% formic acid (buffer A) and 80% ACN in 0.1% formic acid (buffer B). The peptides were separated by a linear gradient of buffer B from 5% up to 95% for 180 min with a flow rate of 300 nl/min. The LC was coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific). The Q Exactive Plus was operated in data-dependent mode with MS scans acquired at a resolution of 70,000, an ion target value of 1e6, and maximum ion injection time for the MS scan was set to 250 ms. Up to 15 most abundant isotope patterns with charge ≥ 2 from the survey scan were selected for MS/MS. An isolation window of 2.0 m/z and higher energy collisional dissociation (HCD) with normalized collision energies of 27% was applied. The maximum ion injection time for the MS/MS scans was set to 100 ms and the ion target value to 1e5. Repeated sequencing of peptides was kept to a minimum by dynamic exclusion of 40 s.^[13-14]

Data analysis Measured MS data were processed using FlexAnalysis (Bruker Daltonics). MS/MS data acquired with the Q Exactive was searched the human I κ B α FASTA using Mascot. Search parameters included two missed cleavages from trypsin proteolysis and fixed modification of cysteine residues. Variable modifications included oxidation (M), and phosphorylation (S and T). The ion-score cutoff was less than zero or 10^{-5} at significance threshold $p < 0.05$.

Statistical analysis Results were expressed as means \pm standard error of the mean (SEM) or frequency (%). An independent t-test was performed to compare the difference of the means between control and experimental groups. All statistical analysis was done using SPSS version 12.0 (SPSS, Inc., Chicago, IL). A p value of less than 0.05 was considered statistically significant.

Results

1. Analysis of phosphopeptides in I κ B α by AurkC using MALDI-TOF MS

In a previous study [17], the interaction between I κ B α and AurkC was identified using the CUPID system. The result was demonstrated through typical experiments, including co-immunoprecipitation and use of a mammalian two-hybrid system. The interaction between I κ B α and AurkC, which are serine and threonine kinases, leads to I κ B α phosphorylation. Interestingly, although I κ B α is known to be phosphorylated at S32 and S36, AurkC phosphorylates I κ B α only at S32, but the phospho-band was thick and shifted to identify a novel phosphorylation site 17. Thus, we screened the I κ B α phosphorylation site to identify the carcinogenic mechanism of I κ B α -AURKC binding and designed a new I κ B α phosphorylation site with AurkC using MALDI-TOF MS and nanoLC-quadrupole orbitrap MS/MS (Supporting Figure 1). Phospho-I κ B α was enriched to analyze the phosphopeptides using MS. Trifluoroacetic acid and the alpha-cyano-4-hydroxycinnamic acid matrix commonly used for peptide analysis were utilized instead of dissolving the 2,5-dihydroxybenzoic acid matrix in a phosphoric acid (PA) solution. To identify the specific phosphorylation site in I κ B α using AurkC, I κ B α phosphorylated by IKK β and unphosphorylated I κ B α were used as negative controls. We detected 11 of the 22 tryptic peptides in phosphorylated I κ B α using MALDI-TOF MS and also found 3 phosphorylated peptides. The tryptic peptides containing missed cleavages and modified peptides, including oxidized (+16 Da) and carbamidomethylated (+57 Da) I κ B α , are shown with their theoretical molecular weights. The phospho-peptide increased about 80 Da because HPO $_3^-$ binds to serine and threonine. The increase of 80 Da in the T6 (amino acids 30–38) peptide including S32 was observed at m/z 1069.40 (P1). We expected that the original T6 peptide (989.44 Da) would be phosphorylated at S32. However, the original T6 peptide peak was not found because it underwent various modifications and missed cleavages (Supplementary Figure 2 and Table 1). The spectra of I κ B α phosphorylated by AurkC were compared with those of unphosphorylated I κ B α and I κ B α phosphorylated by IKK β . We found a novel peak only in I κ B α phosphorylated by AurkC (Figure 1). m/z 1731.81 (P2) and m/z 2340.15 (P3) were expected to increase about 80 Da each from the T9-10 peptide (amino acids 54–67) and T20 peptide (amino acids 246–264) (Supplementary Figure 2), indicating that I κ B α has a novel AurkC phosphorylation site (Supplementary Table 2). In this study, the phosphopeptide analysis was performed in positive ion detection mode without enrichment.

2. Profiling of phosphopeptides in I κ B α by AurkC using nanoLC-quadrupole orbitrap MS/MS

LC-quadrupole orbitrap MS/MS (LC MS/MS) analysis (Q-Exactive Plus) was performed to confirm the MALDI-TOF results. The peptide mixture was generated by an in-gel digestion process and then dissolved in 2% PA. LC MS/MS was performed to separate the tryptic peptides and identify the separated peptides. The Mascot data search program was operated under tryptic mis-cleavage 2, methionine oxidation, and serine/threonine phosphorylation conditions. The LC MS/MS data were matched to 83% of the sequence of I κ B α , except that the largest molecular weight peptide (265–314; 5848.57 Da) and short LTL peptide (315–317; 346.23 Da) from the C-terminal region were produced by trypsin. Moreover, 63 phosphopeptides obtained from the Mascot search results had the expected cut-off value (10-5). Phosphorylated S32-, S63-, and S262-containing peptides of various lengths were confirmed. In conclusion, we observed S32 (Supplementary Figure 3), S63, and S262 phosphorylation sites in I κ B α using by AurkC (Figure 2). Phospho-S63 and phospho-S262 have not been reported in I κ B α until now.

3. AURK phosphorylates the new phosphorylation site of I κ B α .

In vitro kinase assays were used to confirm phosphorylation of the newly discovered S63 and S262 sites. No anti-S63 or -S262 antibody exists, so an antibody was produced. First, S32 of representative I κ B α was phosphorylated to confirm that I κ B α was phosphorylated by AURK (Figure 3A). AurkC was reacted with inactivated I κ B α . As a result, we confirmed that AurkC phosphorylated I κ B α at S32. Based on these results, I κ B α S63 and S262, which were newly detected by AURK, were phosphorylated (Figure 3B). All three AURK families phosphorylated I κ B α at S63 and S262, as well as at S32. The highest affinity was for AurkA and the lowest was for AurkC. Therefore, AURK confirmed that the I κ B α phosphorylation site was phosphorylated.

4. Ser-63 and 262 mutations of I κ B α inhibit cell proliferation in breast cancer cells

Novel S63 and S262 mutants were constructed by single point mutations; serine was substituted with alanine to confirm the intracellular mechanism (Figure 4A). p65 transcriptional activity was confirmed to examine the effect of S63 and S262 phosphorylation on NF- κ B activity in MDA-MB 231 cells (Figure 4B). p65 transcriptional activity in the S63A and S262A mutants was decreased compared with that in the control, and p65 activity in the S63A mutant was further reduced compared with that in the S262A mutant. Inhibition of NF- κ B activity was closely related to survival of cancer cells, confirming the viability and proliferation of the breast cancer cells. MDA-MB 231 and MCF-7 cells showed decreased tumor cell viability in the S63A and S262A mutants (Figure 4C). Both cell lines decreased significantly, and cell viability was decreased slightly in the S63A mutant compared to the S262A mutant. Tumor cell proliferation was also inhibited by both mutations (S63A and S262A; Figure 4D). However, synergistic effects in the S63A and S262A double mutants were not confirmed in any of the three experiments. Nevertheless, the phosphorylation of I κ B α at S63 and S262 not only replaced I κ B α S32, but also regulated downstream NF- κ B activity (Supplementary Figure 4A).

5. S63A and S262A of I κ B α induced necroptosis in MDA-MB 231 cells

The degree of apoptosis in the mutants was confirmed, as the single point mutation in I κ B α affected the viability and proliferation of the breast cancer cell lines. Apoptosis was measured in each mutant using the MDA-MB 231 cell line (Figure 5A). Cell death increased 3–4 fold in the S63A or S262A mutant compared to the control. However, apoptosis increased in the S63A and S262A groups compared to the control group, but no significant difference was observed between the mutant groups and the double mutant group. Interestingly, necrosis changed more than apoptosis in the cell population. In other words, cells that were dead by apoptosis also underwent propidium iodide staining, but the cell population was confirmed to proceed to Q3-Q1 as well as Q3-Q4-Q2. Therefore, programmed necrosis or necroptosis in the population increased for Q1. Thus, the expression and phosphorylation of RIP3 and MLKL, which are related to necroptosis, were confirmed (Figure 5B). The phosphorylation of RIP3 and MLKL increased 2–2.5 times compared to the control. The mutant group also showed increased expression of cleaved caspase-3 and -8 compared to the control group. Based on these results, S63 and S262 of I κ B α also have important effects, including apoptosis and necroptosis, upon I κ B α phosphorylation.

Discussion

NF- κ B activity is mediated through the phosphorylation and degradation of I κ B α . Phosphorylation of I κ B α is an important intracellular mechanism [8]. I κ B α phosphorylation is induced by a variety of kinases [8, 18]. The most important pathway is the phosphorylation of S32 and S36 by IKK β , which leads to the degradation of I κ B α [8, 18]. Other pathways include the phosphorylation of I κ B α by casein kinase II (CK2), leading to the phosphorylation and degradation of I κ B α at S283, S293, and Thr299.[19, 20] However, there are still unknown I κ B α phosphorylation mechanisms and phosphorylation sites, and many of the biological and oncological functions are unknown. This study identified new phosphorylation sites of I κ B α and suggested its oncological function. I κ B α is phosphorylated by protein-protein interactions between I κ B α and AURK.[5, 17] AURK also regulates cell cycle and cell division. To suppress the proliferation of cancer cells, more powerful inhibition of I κ B α is required, and thus, I κ B α activity is controlled through AURK.[17] The ankyrin domain of I κ B α is required to interact with AURK. I κ B α phosphorylation by AURK differs from I κ B α phosphorylation by IKK family proteins; the I κ B α S36 residue was not phosphorylated by AURK.[17, 21] We identified three novel phosphorylation sites at S63, S160, and S262 using MALDI-TOF MS and nanoLC-MS/MS.[22, 23] However, in vitro phosphorylation assays showed modification at Ser63 and Ser262. Ser160 was not only weakly phosphorylated by an in vitro kinase but also showed decreased antibody specificity (data not shown). Ser63 and Ser262 of I κ B α were confirmed to be phosphorylated as much as Ser32 by AURK in the in vitro kinase assay.

Minor phosphorylation is known to be inferior to most major phosphorylation mechanisms, such as biological or oncological functions. However, the sites found in this study (S63 and S262) were less affected than S32 (Supplementary Figure 4), but phosphorylation was induced by AURK, which is not a known kinase. We also confirmed that the proliferation and viability of breast cancer cells were decreased in lines carrying two amino acid substitutions: S63A and S262A. This finding suggests that substituting

alanine suppressed the degradation of I κ B α and inhibited breast cancer cell proliferation and survival. Although p65 activity was inhibited by mutants, it was confirmed that the effect of combination was insignificant. Structurally, both S63 and S262 of I κ B α are closely located at the interacting interface between two proteins such as I κ B α and NF- κ B p65, so it is thought that synergy in combination may not be seen (<https://www.rcsb.org/3d-view/1IKN>). We performed a FACs-apoptosis assay to confirm reduced breast cancer cell survival. Both the S63A and S262A groups showed increased early and late apoptosis. Interestingly, there was a larger necrotic region than apoptotic region. Based on these results, we confirmed the expression and phosphorylation of RIP3 and MLKL, indicating necroptosis and programmed necrosis, in S63A and S262A. As a result, the phosphorylation of RIP3 and MLKL was increased in both S63A and S262A of the I κ B α groups. I κ B α , a major cellular regulator, is regulated through phosphorylation by various kinases.[11, 12] In particular, avoiding necroptosis or apoptosis through phosphorylation by AURK in cancer cells is an important regulatory mechanism.[24, 25] The newly identified I κ B α phosphorylation site is an important part of the regulation of I κ B α , including for necroptosis or apoptosis. Although there are several tumor regulatory mechanisms that have not yet been identified, the discovery of this phosphorylation mechanism is essential for understanding the regulation of breast cancer.

Conclusions

We identified novel phosphorylation site of I κ B α by AURK, and its site was related to apoptosis. But, the role of apoptosis in the new phosphorylation site was insufficient compared to S32 of I κ B α . However, based on the results of FACs, it was newly identified as a site involved in necroptosis.

Declarations

Ethics approval and consent to participate

- No results involved for human or human tissues or data

Availability of data and materials

- The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
- All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Competing interest: Nothing to disclosure

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Authors' contributions

SHC and YHC designed the experiment and analyzed data. SHC, HY and SHY performed screening and analyzed proteomic experiments. SHC, HY and JHP performed other in vitro experiments. EHH and SGC provided technical support for in vitro. SHC, SGC and YHC wrote the manuscript and confirmation. All authors read and approved the final manuscript.

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Abbreviations

MS Mass spectrometry, **MALDI** Matrix-assisted laser desorption/ionization, **TOF** Time of flight. **LC** Liquid Chromatography, **IP** Immunoprecipitation, **I κ B α** nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha, **AURK** Aurora Kinase, **IKK** I κ B kinase, **TEV protease** Tobacco etch virus protease, **ACN** Acetonitrile, **SDS-PAGE** Sodium Dodecylsulfate Polyacrylamide Gel electrophoresis, **2,5-DHB** 2,5-dihydroxybenzoic acid, **PA** phosphoric acid, **TUNEL** terminal deoxynucleotidyl transferase dUTP nick end labeling, **PPI** Protein-Protein Interaction

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Figures

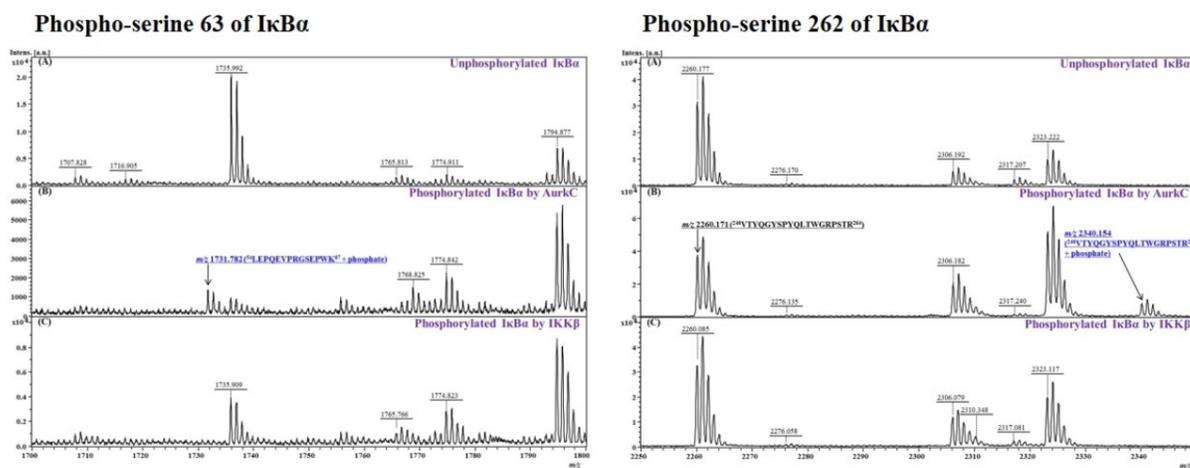


Figure 1

Identification of phosphorylation of serine 63 and 262 on IκBα using MALDI-TOF MS A) Newly found phospho-serine 63 of IκBα. Comparison of Mass range expansion of 1700-1750 m/z regions from the MALDI mass spectra of (A) inactivated IκBα; (B) IκBα activated by AurkC; (C) IκBα activated by IKKβ. Mass spectrum of IκBα activated by AurkC indicated exclusive peaks. Peaks were 54-67 peptide including phospho-serine 63 amino acid from IκBα. B) Novel phospho-site serine 262 amino acid from IκBα.

Comparison of MALDI MS spectra of (A) non-activated IκBα; (B) IκBα activated by AurkC; (C) IκBα activated by IKKβ tryptic digestion peptide between m/z 2250 and 2350 was shown. Unique peaks found m/z 2340.154 in (B). Peaks were 246-264 peptide including unknown phosphorylated amino acid from IκBα. A peptide present five serine and threonine.

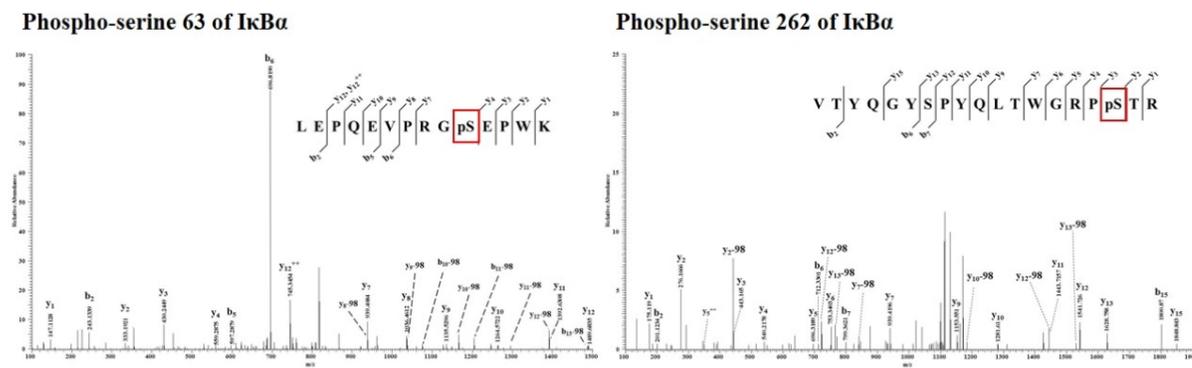
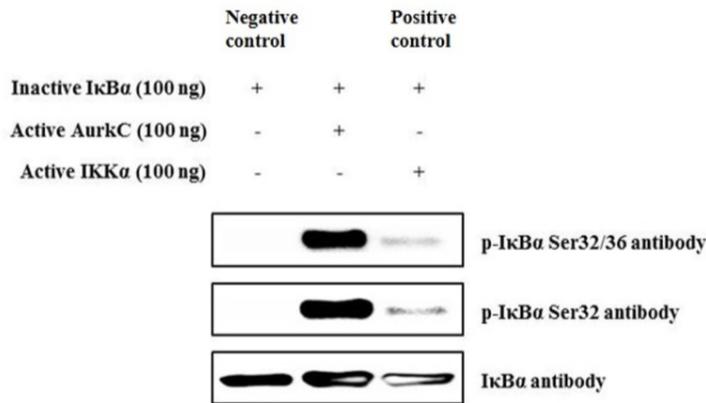


Figure 2

Identification of phosphorylation of serine 63 and 262 on IκBα using nanoLC-quadrupole orbitrap MS/MS

A) Novel phosphorylated serine 63 residue of IκBα was activated by AurkC. LC-quadrupole orbitrap MS/MS spectra of precursor ion m/z 866.4101 (charge state 2+) representing the phospho-peptide of sequence 53-LEPQEVPRGpSEPWK-68. The presence of a yn, yn-98 and bn-98 series identifies phosphorylation at serine 63. B) Novel phosphorylated serine 262 of IκBα activated by AurkC. LC-MS/MS spectra of precursor ion m/z 1170.5469 (charge state 2+) representing the phosphopeptide of sequence 245-VTYQGYSYPYQLTWGRPpSTR-265.

A



B

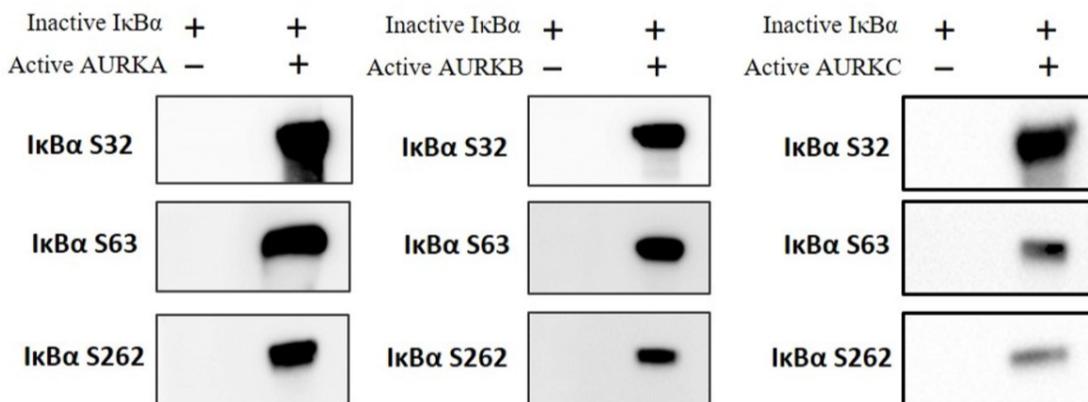


Figure 3

Phosphorylation of I κ B α by Aurora Kinase A) I κ B α was phosphorylated by AURKC. In vitro phosphorylation of I κ B α with AURKC or IKK α kinase protein was performed by phospho-serine 32 amino residue antibody and phospho-serine 32/36 amino residues antibody. I κ B α was phosphorylated by both AURKC and IKK α . B) In vitro phosphorylation of I κ B α with all AURK proteins were performed by phospho-serine 63 amino residue antibody and phospho-serine 262 amino residues antibody. I κ B α was phosphorylated by active all AURK proteins.

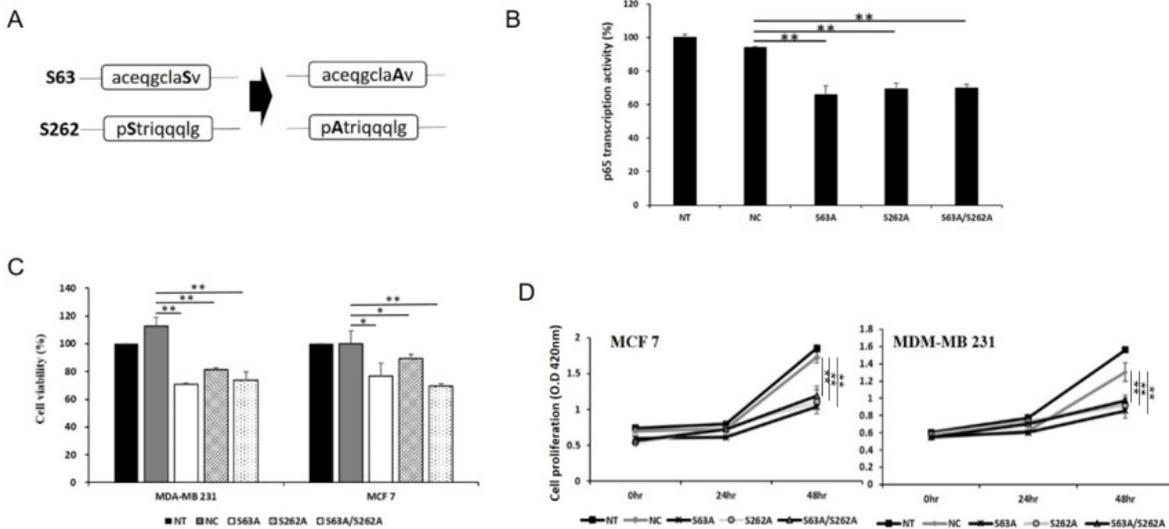


Figure 4

Ser-63 and 262 mutations of I κ B α inhibit cell proliferation in breast cancer cells. Novel S63 and S262 mutants were constructed by single point mutations; serine was substituted with alanine to confirm the intracellular mechanism (Figure 4A). p65 transcriptional activity was confirmed to examine the effect of S63 and S262 phosphorylation on NF- κ B activity in MDA-MB 231 cells (Figure 4B). Inhibition of NF- κ B activity was closely related to survival of cancer cells, confirming the viability and proliferation of the breast cancer cells. MDA-MB 231 and MCF-7 cells showed decreased tumor cell viability in the S63A and S262A mutants (Figure 4C). Tumor cell proliferation was also inhibited by both mutations (S63A and S262A; Figure 4D).

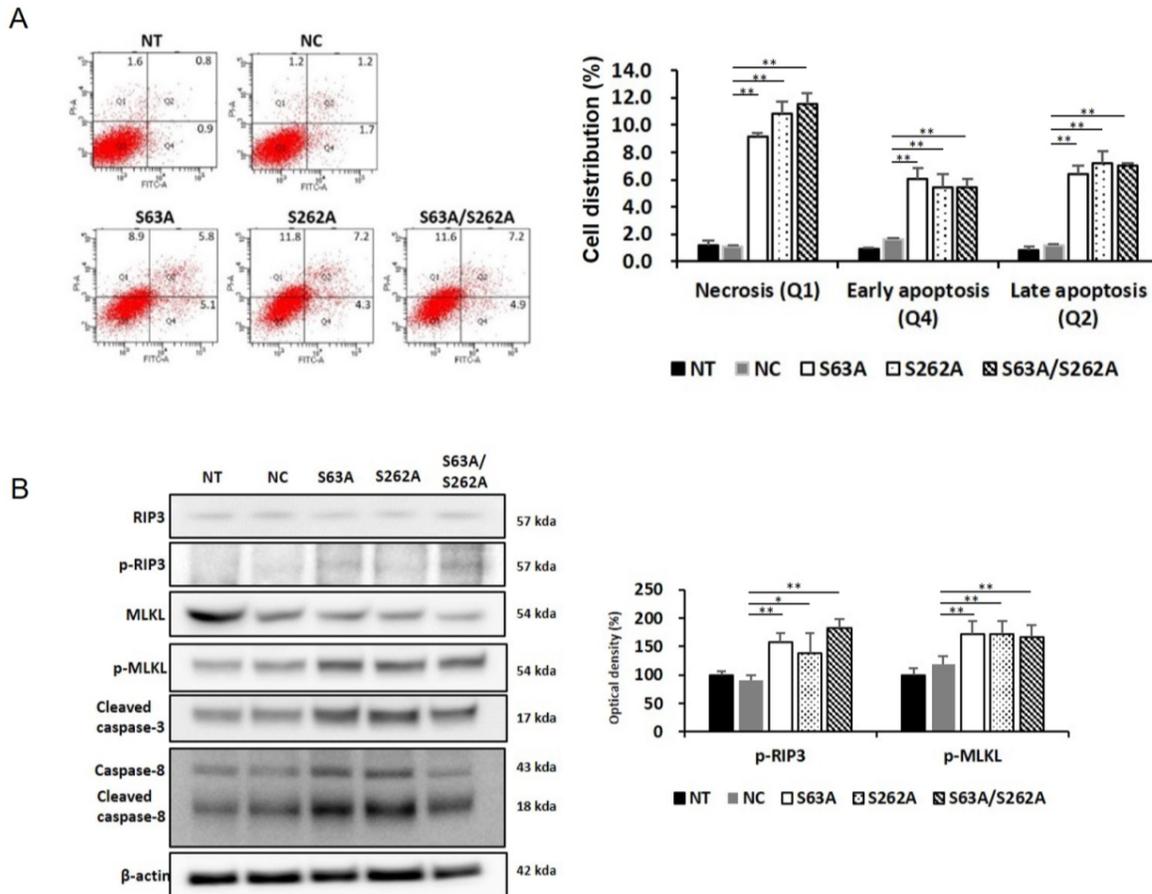


Figure 5

Phosphorylation of Ser-63 and 262 of I κ B α plays a crucial role in necroptosis. FACS-annexin V-staining was performed to measure apoptosis in MDA-MB 231. A) Compared with the control group, the distribution of early and late apoptosis was increased and the distribution of necrosis was increased in S63A and S262A. B) Compared with the control group, expression of phospho-MLKL and phospho-RIP3

was increased in S63A and S262A, and expression of cleaved caspase 3 and caspase 8 was also increased.

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