Published in final edited form as:

Cancer Res. 2006 July 1; 66(13): 6722–6731.

Protein Kinase CK2 Mediates Inhibitor-Kappa B Kinase and Aberrant Nuclear Factor- κ B Activation by Serum Factor(s) in Head and Neck Squamous Carcinoma Cells

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Abstract

We previously showed that the signal transcription factor Nuclear Factor-kappaB (NF- κ B) is aberrantly activated and that inhibition of NF- κ B induces cell death and inhibits tumorigenesis in Head and Neck Squamous Cell Carcinomas (HNSCC). Thus, identification of specific kinases underlying the activation of NF- κ B could provide targets for selective therapy. Inhibitor KappaB Kinase (IKK) is known to activate NF- κ B by inducing N-terminal phosphorylation and degradation of its endogenous inhibitor, Inhibitor- κ B (I κ B). Casein Kinase II (CK2) was previously reported to be over expressed in HNSCC cells, and to be a C-terminal I κ B kinase, but its relationship to NF- κ B activation in HNSCC cells is unknown. In this study, we examined the contribution of IKK and CK2 in the regulation of NF- κ B in HNSCC *in vitro*. NF- κ B activation was specifically inhibited by kinase dead mutants of the IKK1 and IKK2 subunits or siRNA targeting the β subunit of CK2. CK2 and IKK kinase activity, as well as NF- κ B transcriptional activity, were shown to be serum responsive, indicating that these kinases mediate aberrant activation of NF- κ B in response to serum factor(s) *in vitro*. Recombinant CK2 α was shown to phosphorylate rIKK2, as well as to promote immunoprecipitated IKK complex from HNSCC to phosphorylate the N-terminal S32/S36 of I κ B α . We conclude that the aberrant NF- κ B activity in HNSCC cells in response to serum is partially through a novel mechanism involving CK2 mediated activation of IKK2, making these kinases candidates for selective therapy to target the NF- κ B pathway in HNSCC.

Keywords

Casein Kinase 2; Inhibitor-KappaB Kinase; Nuclear Factor kappaB; squamous cell carcinoma; Serum

Introduction

The Nuclear Factor- κ B (NF- κ B/Rel) proteins, a family of nuclear transcription factors and their inhibitors (I κ Bs), are normally involved in regulating a wide range of intracellular processes including response to oxidative stress, inflammation, growth factors, injury, and programmed cell death (1). Aberrant NF- κ B activation has been detected in a variety of cancers, including head and neck squamous cell carcinomas (HNSCC) (1–3). We discovered that NF- κ B1/RelA (p50/p65) activation is increased with tumor progression in murine SCC, and in cell lines and tumor specimens from patients with HNSCC (4–8). Increased nuclear staining of the phospho-activated form of p65 has recently been demonstrated in the majority of high grade squamous dysplasias and HNSCC specimens from patients, and correlated with decreased

survival, supporting the wider importance of NF- κ B in tumor development and clinical outcome of HNSCC (9). We have also demonstrated that NF- κ B is an important modulator of the altered pattern of gene expression and malignant phenotype (10), indicating that NF- κ B activation is an important target for therapy. Specific inhibition of NF- κ B by over-expression of an I κ B α with S32/S36 phosphorylation site mutations (I κ B α M) restored altered gene expression, and inhibited SCC cell proliferation, survival, migration, angiogenesis, tumorigenesis and radiation resistance in experimental models (7,10,11). Bortezomib, an inhibitor of proteasome and I κ B degradation, had similar effects in preclinical and a recent phase I clinical study (8,12). However, the extent of inhibition of the proteasome, NF- κ B, and tumor response was limited by the dose of bortezomib achievable without significant side effects (8). Thus, identification of the specific kinases underlying the signal phosphorylation of I κ B α and activation of NF- κ B could provide more selective targets for therapy in patients with HNSCC.

The eponymous Inhibitor-KappaB Kinase (IKK) plays a critical role in activating NF- κ B-mediated events in cell survival and the immune response (2). The classical IKK complex and pathway is comprised of IKK1 (IKK α), IKK2 (IKK β) and NEMO (IKK γ) subunits. An alternate pathway involving IKK1 has also been described (2). In normal cells, the classical IKK complex plays a critical role in integrating responses to various stimuli, resulting in IKK2-mediated phosphorylation of S32/S36 in the N-terminus of I κ B α , and NF- κ B activation. Casein Kinase II (CK2) is another stress activated protein kinase that participates in the transduction of signals that promote cell growth and survival (13), and has been implicated in NF- κ B activation (14–18). In distinction to IKK2, CK2 has been shown to directly phosphorylate the C-terminal PEST domain of I κ B α , and has been recently shown to be a C-terminal I κ B α kinase responsible for ultraviolet light (UV) induced NF- κ B activation (14–16). CK2 may also promote transactivation by phosphorylation of the RelA subunit of NF- κ B (17,18). CK2 is over-expressed and activated in HNSCC cell lines and tumor specimens, and associated with poor prognosis (19,20). Furthermore, anti-sense RNA targeting CK2 α was found to inhibit proliferation of an HNSCC cell line (21), similar to our findings with inhibition of NF- κ B. These observations suggested a potential relationship and a role for CK2 in upstream signaling and activation of NF- κ B in HNSCC.

The signals mediating NF- κ B activation in HNSCC have not been elucidated. CK2, IKK and NF- κ B may be activated in response to a variety of cytokines, growth, and serum factors (13, 17,18,22–26). In the present study, we examined the contribution of CK2, IKK and serum to aberrant NF- κ B activity in HNSCC.

Methods and Materials

Cell Lines

HNSCC cell lines from the University of Michigan Squamous Cell Carcinoma (UM-SCC) series were obtained from Dr. T.E. Carey, University of Michigan and previously described (27). All UM-SCC cell that are used for this study were maintained in MEM medium (Invitrogen, Carlsbad, CA) with 10% FBS. Supplemented Serum Free Medium (SFM) was the same MEM medium supplemented with Non-Essential Amino Acids, Insulin-Transferrin-Selenium-G, Vitamin Solution, Fibronectin, (Invitrogen, Carlsbad, CA), and trace elements (Biofluid, Camarillo, California). Human keratinocytes (HEKa) were purchased and maintained in supplemented 154 CF Medium with 200 mM CaCl₂ (Cascade Biologics Inc. Portland, OR). All cells were maintained at 37°C in 5% CO₂ atmosphere.

Reagents

Anti-IKK2 and CK2 β antibodies were purchased from BD Pharmingen (San Jose, CA). Anti I κ B α , IKK1 and -NEMO antibodies were purchased from Santa Cruz (Santa Cruz, CA). Wild type and mutant I κ B α -GST fusion proteins and pcDNA3-IKK vectors were kind gifts from Dr. Keith Brown, National Institute of Allergy and Infectious Disease, NIH.

Western Blot Analysis

10 μ g protein of whole cell lysates were used for each assay with standard Immunoblotting procedures. Total proteins that were transferred onto the membrane were stained and quantified for equal loading control and for normalizing the intensities of the detected specific protein bands.

Electro Mobility Shift Assay (EMSA)

Nuclear extracts were obtained by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). 5 μ g nuclear extract was used for EMSA with ³²P-NF- κ B response element oligonucleotide (Sense: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', antisense: 3'-TCA ACT CCC CTG AAA GGG TCC G-5', Promega Cat#: E3292, Madison, WI). ³²P-OCT-1 was used as a positive control for loading.

NF- κ B Reporter Assay

Cells were transfected by jetPEI complexed with control or cis-pNF- κ B-luc cis-reporter plasmids (PathDetect® in Vivo Signal Transduction Pathway *cis*-Reporting Systems NF- κ B (5 $\times$) [TGGGGACTTTCCGC], Cat#: 219077, Stratagene, San Diego, CA, <http://www.stratagene.com/manuals/219073.pdf>). After 24 hours, luciferase activities were measured with a dual-luciferase system from Promega (San Luis Obispo, CA) using a Vector II spectrophotometer (Perkin Elmer, Boston, MA). pCIS-CK plasmid was employed as a negative control for the pNF- κ B-luc cis-reporter plasmid while pFC-MEKK plasmid (both obtained from Stratagene, San Diego, CA) was co-transfected with pNF- κ B-luc cis-reporter plasmid to serve as a positive control. The firefly-luciferase activities of triplicate samples were averaged following normalization by corresponding renilla-luciferase activity co-transfected in the same wells. The NF- κ B-luciferase activity is presented as the ratio of NF- κ B-luc/CK-luc.

CK2-siRNA Transfection

Cells were transfected with CK2 β -Stealth-RNAi (Sense: 5'-CCAGCAACUUAAGAGCCAGUCAA, Anti-Sense 5'-UUGACUGGGCUCUUGAAGUUGACGG), a corresponding Scramble Stealth RNAi control (Sense 5'-CCACAACUUGAACGAACCUGGCCAA, Anti-Sense 5'-UUGGCCAGGUUCGUUCAAGUUGUGG) (both designed by Invitrogen BLOCK-iT™ RNAi Designer <https://rnaidesigner.invitrogen.com/rnaiexpress/> Invitrogen, Carlsbad, CA), or a universal negative control siRNA (Cat# 4611, Ambion, Austin, TX). and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 hours followed by evaluation assays. Transfection efficiency and cell viability were monitored by FITC labeled RNA oligo, DAPI and Dead Cell Dye respectively (Invitrogen, Carlsbad, CA).

mRNA Quantification

I κ B α , NFKB2/p100 and CK2 β mRNA levels from the cells were quantified by QuantiGene Assay kit with probe sets designed to target each of these mRNAs specifically (Genospectra, Fremont, CA). The results were normalized to corresponding 18sRNA levels.

IKK Kinase Assay

Cells were lysed in 25mM HEPES, 150mM NaCl, 1% Triton X100, 10% glycerol, 5mM EDTA, 2mM DTT, and protease and phosphatase inhibitor cocktails. IKK was immunoprecipitated (IP) from cell lysates by polyclonal anti-NEMO antibody (Santa Cruz, Santa Cruz, CA). Kinase assay was performed in kinase buffer (20mM HEPES, pH7.5, 10mM MgCl₂, 1mM DTT, 1mM sodium ortho vanadate, 25mM β -glycerol phosphate) by incubating IKK complex with either N-terminal 72 amino acid of wild-type I κ B α (S32/S36)-GST or mutated I κ B α (S32G/S36A)-GST fusion proteins as substrate at 30°C for 30 minutes. The products from the kinase reaction were separated on a 4–12% SDS Gradient gel and, then transferred onto a nitrocellulose membrane. IKK kinase activity was quantified by the intensity of the ³³P-I κ B α -GST band detected by autoradiography and normalized as a ratio to the corresponding quantified protein levels of I κ B α and, then, IKK2 detected from immunoblot analysis on the same membrane.

CK2 Kinase Assay

Cell lysates were prepared as described above for IKK Kinase Assay. CK2 kinase activity was measured by using a CK2 Kinase Assay Kit with a CK2 specific peptide substrate (Upstate, Charlottesville, VA; for details refer to <http://www.upstate.com/img/coa/17-132-18642.pdf>). 20 μ g total protein of the lysate from each reaction was incubated along with a PKA inhibitor and [γ ³³P]-ATP in assay dilution buffer for 10 minutes at 30°C. The phosphorylated substrate is then separated by P81 phosphocellulose paper and quantified by a scintillation counter. Active CK2 enzyme was used as positive control. Spike assay was also employed to ensure accuracy.

Results

Aberrant Activation of NF- κ B in UM-SCC cell lines

We previously showed that NF- κ B is activated in several human HNSCC cell lines from the University of Michigan Squamous Cell Carcinoma (UM-SCC) series (6,7). To select representative cell lines for further analysis, we compared NF- κ B DNA binding activity in a panel of 9 UM-SCC cell lines derived from 7 patients, and in non-malignant human keratinocytes (HEKa). Increased NF- κ B DNA binding activity was detectable in 8/9 UM-SCC cell lines from 6/7 patients relative to HEKa (Fig. 1A), similar to the frequency of increased NF- κ B activation observed in HNSCC tumor relative to normal squamous mucosa specimens (9). To determine if variations in DNA binding are reflected by variations in functional activation, NF- κ B luciferase reporter activity was compared in UM-SCC-1, -6, -9 and -11A and B cell lines. Variable NF- κ B reporter activities were observed in these cells (Fig. 1B). Thus, UM-SCC cell lines demonstrated aberrant NF- κ B activation, with a prevalence and variability similar to that reported for HNSCC tumor specimens (8,9).

UM-SCC-6 showed significantly higher NF- κ B reporter activity when compared to the other cell lines ($p < 0.01$) while UM-SCC-9 showed significantly lower NF- κ B reporter activity compared to the other cell lines ($p < 0.05$ between UM-SCC-11B and UM-SCC-9, and $p < 0.01$ to the other three cell lines). Protein levels of I κ B α , a protein both induced by and degraded with activation of NF- κ B, were compared between UM-SCC-6 and UM-SCC-9 cells to determine the relative differences in NF- κ B activation (Fig 1 C). Cells were treated with 50 μ g/ml cycloheximide for variable times as indicated. I κ B α levels were quantified by the intensity of the band on immunoblot and normalized to total protein levels detected on the membrane. Fig. 1C shows that the endogenous I κ B α protein expression level in UM-SCC-6 cells is ~1.8 fold that in UM-SCC-9 cells, consistent with the relatively greater activation of NF- κ B detected in UM-SCC-6 cells. The data are representative of 2 independent experiments. UM-SCC-6 cells also showed a ~50% shorter I κ B α half-life ($T_{1/2}$ ~1.5 vs. 3 hrs, Fig. 1C), and

2-fold difference in IKK kinase activity for phosphorylation of S32/S36 I κ B α compared to UM-SCC-9 cells (Fig1 D). These data confirm that UM-SCC-6 cells exhibit greater NF- κ B activity, and signal kinase activation by IKK than UM-SCC-9 cells. Based on all of these observations, we chose UM-SCC-6 and 9 for further studies based upon the range of strong and weak NF- κ B activation detected in these two cell lines.

The IKK1 and 2 Subunits Contribute to NF- κ B Activation in UM-SCC Cells

The IKK complex has previously been shown to mediate phosphorylation of I κ B α and regulate canonical activation of NF- κ B by proinflammatory cytokines (13,28). The role of IKK in aberrant NF- κ B activation in HNSCC was examined by co-transfection of amino acid substituted dominant positive (S176E/S180E, IKK1^{EE} and S177E/S181E, IKK2^{EE}), dominant negative (S176A/S180A, IKK1^{AA} and S177A/S181A, IKK2^{AA}), Kinase dead IKK (K44A, IKK1^{KA} and IKK2^{KA}), or wild type IKK1 or IKK2 vectors together with cis-pNF- κ B-luc in UM-SCC-6 cells (28). Fig. 2 A and B show that constitutively active IKK1^{EE} and IKK2^{EE} induced over a 20-fold increase in NF- κ B reporter activity compared to that of control cells ($p < 0.001$). Co-transfection and expression of additional wild-type IKK1 or IKK2 induced a ~1.5 ($p < 0.05$) and 4-fold ($p < 0.001$) increase of NF- κ B activity respectively. Thus, IKK^{EE} and wild-type both increased NF- κ B activity, serving as a positive control for the transfection and responsiveness of the cells. In contrast, the IKK2^{KA} partially reduced the NF- κ B reporter activity by ~65% ($p < 0.001$) while IKK1^{KA} reduced activity by ~50% ($p < 0.001$). The partial inhibition of NF- κ B reporter activity observed with either kinase dead IKK1^{KA} or IKK2^{KA} in UM-SCC-6 cells is consistent with the partial ~50–80% inhibition we have observed in repeated experiments with IKK1^{KA} or IKK2^{KA}, IKK1 or IKK2 siRNA, or IKK2 inhibitor PS-1145 in UM-SCC-11A and 11B (L. Nottingham, J. Ricker, unpublished observations). The contribution of both IKK1 and IKK2 to NF- κ B activation is also supported by their independent roles in processing of p100 to p52 (29) and degradation of I κ Bs (28), both of which were observed after cycloheximide treatment in UM-SCC-6 cells (Fig. 1C and data not shown). In addition, co-transfection of the phosphoacceptor mutants IKK2^{AA} did not inhibit NF- κ B activity ($p > 0.10$) and that of IKK1^{AA} increased NF- κ B activity ($p < 0.05$) in UM-SCC-6 (Fig. 2 A and B), suggesting that activation of these IKKs and NF- κ B may occur via a mechanism other than that previously reported for classical NF- κ B activation by TNF and IL-1 (28).

CK2 contributes to Aberrant NF- κ B activation in UM-SCC cells

CK2 has been reported to induce NF- κ B activation (14–18). Elevated CK2 activity has been demonstrated in HNSCC and linked to cell proliferation and patient survival (19–21). Treatment of UM-SCC cells with apigenin, an agent that inhibits CK2 kinase activity (30), strongly inhibited NF- κ B reporter activity in all 4 UM-SCC cell lines (Fig. 3A, UM-SCC-6 and -11A $p < 0.001$, UM-SCC-9 and -11B $p < 0.05$). Depletion of the regulatory subunit CK2 β has previously been shown to inhibit CK2 mediated activation of NF- κ B by UV light (16). To examine whether CK2 specifically contributes to aberrant NF- κ B activity, small interference RNA (siRNA) targeting CK2 β was used. Transfection of UM-SCC-6 and UM-SCC-9 cells with human CK2 β siRNA resulted in a ~90% decrease of CK2 β mRNA in UM-SCC-6 cells and ~75% decrease of that in UM-SCC-9 cells (Fig. 3B for UM-SCC-6 cells $p < 10^{-9}$ and C, for UM-SCC-9 cells $p < 10^{-5}$). The decreased expression of CK2 β resulted in a corresponding 82% reduction in NF- κ B reporter activity in UM-SCC-6 (Fig. 3D, $p < 0.05$). We also observed an ~60% reduction of NF- κ B reporter activity in UM-SCC-9 cells, but this did not reach the level of statistical significance, with the variance observed at the low level of activation detected in this cell line (Fig. 3E, $p = 0.09$). The inhibition of NF- κ B reporter activity observed was not due to non-specific inhibition of mRNA expression by siRNA or transfection, as no significant reduction was observed with unrelated or scrambled control siRNAs. Consistent with CK2 β siRNA inhibitory effect on NF- κ B reporter activity, we also observed a decrease in expression of NF- κ B inducible genes I κ B α (58%, $p < 10^{-6}$) and NFKB2 (68%, $p < 10^{-7}$) in

anti-CK2 β siRNA transfected UM-SCC-6 cells, when compared to that of CK2 β scramble control siRNA transfected cells (Fig 3 F). Comparable cell viability among different siRNA treatments during the assay was monitored by DAPI staining and propidium iodide staining to exclude the possibility that effects were the result of cell death (data not shown). Together with data using inhibitor apigenin, these siRNA data indicate that CK2 is involved in increased NF- κ B activation and expression of its target genes in UM-SCC 6 cells.

Aberrant NF- κ B Activation by UM-SCC Cells Occurs in Response to Serum

Aberrant NF- κ B activation in response to growth factor receptors has previously been observed in breast cancer (31,32). To explore the contribution of serum to the aberrant activation of NF- κ B in UM-SCC cells, we examined the effects upon NF- κ B reporter activity of replacing FBS with supplemented serum-free media (SFM). A panel of four UM-SCC lines newly changed to SFM (SFM-new) or serially adapted to SFM (SFM-adp) before transfection, each showed a progressive reduction of NF- κ B reporter activity when compared to cells grown in 10% FBS that is not heat-inactivated (FBS-NHI) (Fig. 4A, $p < 0.01$ for both SFM-new and SFM-adp comparing to FBS-NHI; $p < 0.01$ between SFM-new and SFM-adp). Accordingly, ~50% decrease of endogenous I κ B α mRNA level was also observed in SFM-adp UM-SCC-6 cells comparing to that in FBS-NHI cells ($p < 10^{-5}$, data not shown). Heat-inactivation of FBS (FBS-HI) to 56°C also demonstrated a significant decrease in NF- κ B reporter activity (Fig. 4A, $p < 0.01$ when compared to FBS-NHI). The NF- κ B reporter activities from the cells that were grown in FBS-HI are also significantly higher than those in SFM-adp ($p < 0.01$), but were not statistically different when compared to those grown in SFM-new ($p > 0.05$). Co-transfection and expression of MEKK, an up-stream activator of NF- κ B, increased NF- κ B reporter activity under each of the conditions, indicating that NF- κ B in UM-SCC cells remained responsive to signal activation (data not shown). The results shown here represent the average of 4 experiments. Two different sources of serum were employed to exclude the possibility of single batch effects of the serum. Together, these results indicate that NF- κ B activation is increased in UM-SCC cells in response to serum, and that these serum components are temperature sensitive.

Increased CK2 kinase activity in response to serum in UM-SCC-6 cells

CK2 is known to mediate signal responses to serum (22–26) and we observed that the extent of reduction of NF- κ B reporter activity, in the absence of serum, was similar to that observed following inhibition of CK2 by either apigenin or CK2 β siRNA. This led to the hypothesis that CK2 may mediate the activation of NF- κ B by serum component(s). To examine this hypothesis, we first compared CK2 kinase activities by a CK2 specific peptide substrate from UM-SCC-6 cells that were cultured either in FBS or SFM. CK2 activity from cell lysate that were cultured in SFM was significantly reduced by 50% compared to that in FBS ($p < 0.001$ Fig 4B). Applying apigenin resulted in significant further reduction of CK2 kinase activity in both of the cultures ($p < 0.05$) (Fig 4B) and strongly inhibited a positive control with recombinant CK2 α alone. A PKA inhibitor was employed to block non-specific kinase activity in these assays. Spiking cell lysates with a known amount of rCK2 α demonstrated a ~70% rate of recovery of CK2 activity by the assay (data not shown). Additionally, we also detected an statistically significant ~30% decrease of CK2 β ($p < 0.01$, Fig 4 C) and an ~50% decrease of I κ B α ($p < 10^{-6}$, data not shown) mRNA expression level in the cells that were cultured under SFM compared to that under FBS-NHI, suggesting that a serum dependent decrease in CK2 expression contributes to reduced NF- κ B activity and expression of target gene I κ B α . The differences in CK2 kinase activity between SFM and FBS indicates that CK2 activity is increased in response to serum in UM-SCC-6 cells and suggests that CK2 may play an important role in mediating the increased NF- κ B activity in response to serum.

Increased IKK activity in response to serum in UM-SCC-6 cells

In Fig. 2, we showed that co-transfection of kinase dead IKK2^{KA} inhibited NF- κ B activity in UM-SCC-6 cells by ~65% (Fig. 2A) and that SFM also reduces NF- κ B activity in these cells (Fig. 4A). We next examined the effect of serum on IKK kinase activity in UM-SCC-6 cells. IKK kinase activity from whole cell lysate of UM-SCC-6 cells cultured with FBS or SFM was measured as described in methods. A ~50% decrease of IKK kinase activity was observed from cells that were cultured in SFM when compared to that in FBS in two independent experiments (Fig. 4D and data not shown). The change in IKK kinase activity is consistent with the magnitude of contribution of IKK2 to NF- κ B activation observed in Fig. 2. Specific phosphorylation of S32/S36 I κ B α , the phospho-acceptor sites for IKK2, was ensured by comparison with a negative control S32G/S36A I κ B α mutant substrate (Fig. 4D). Thus, our data indicates that IKK mediates signal phosphorylation of I κ B α , and activation of NF- κ B in response to serum by UM-SCC-6 cells.

CK2 promotes IKK kinase activity

Since the data above indicate that both CK2 and IKK mediate the response to serum and contribute to NF- κ B activity in UM-SCC-6 cells, we wondered if CK2 could modulate IKK2 mediated phosphorylation of I κ B α . In this regard, protein sequence analysis revealed 11 putative CK2 phosphorylation consensus motifs in IKK2. Based on prediction algorithms, 6 of these motifs had a score (probability to be phosphorylated by CK2) higher than 0.92 on a 0 to 1 scale (data not shown). This analysis suggested that IKK2 could be a potential substrate of CK2 and, hence, IKK kinase activity could be regulated by CK2. We examined whether IKK2 is a substrate of CK2 in an *in vitro* CK2 kinase assay by employing rCK2 α and rIKK2 (Fig. 5A). In this assay, we used 0.5 and 5 ng of rCK2 α with rIKK2 (200ng/reaction). Since CK2 can use both ATP and GTP, to ensure the CK2 specificity, we used GTP rather than ATP as a phospho-donor. Our result showed a low level of auto-phosphorylation by IKK2, which may be caused by the high concentration of rIKK2 used. This was confirmed by comparing GTP to ATP as a phospho-donor, which showed more dramatic auto-phosphorylation by IKK2 itself (data not shown). On top of this IKK2 auto-phosphorylation, we detected an rCK2 α dose-dependent increase in phosphorylation of rIKK2. In the presence of 0.5ng and 5ng rCK2 α , a ~40% and 2.2 fold increase of normalized phosphorylated IKK2 was observed when compared to control. Apigenin completely suppressed CK2 α phosphorylation of IKK2. The result shown here is an average of two parallel measurements. Thus, these data provide direct evidence that IKK2 is a substrate of CK2.

To determine if CK2 could modulate IKK2 kinase activity as part of the IKK complex, anti-NEMO antibody immunoprecipitated IKK complexes were incubated with different amounts of catalytically active rCK2 α and GTP under the conditions optimized for CK2 kinase assay, followed by washing out of CK2, prior to the IKK kinase assay. Since the substrate I κ B α -GST fusion protein used only contains the N-terminal 72 amino acids, the assay excludes that CK2 phosphorylation is due to the I κ B α C-terminal PEST domain. Again S32G/S36A mutant I κ B α -GST was used as a negative control to ensure IKK specific phosphorylation of the S32/S36 of the N-terminus I κ B α . The kinase activities were normalized to protein levels of I κ B α and IKK2. Fig. 5B shows the rCK2 α dose dependent increase of IKK kinase activity detected as IKK mediated phosphorylation of wild type, but not S32G/S36A mutant I κ B α -GST. A 13% and 60% increase of IKK kinase activities from those that were preincubated with 0.5ng and 5ng rCK2 α respectively was observed compared to that of non-treated IP-IKK complexes. When the IP-IKK complex was incubated with 5ng rCK2 α in the presence of 40 μ M apigenin, the CK2 α induced IKK kinase activity was completely blocked. A ~70% increase in IKK kinase activity was detected with saturating amounts of rCK2 α (10ng and 20ng) in an independent experiment (data not shown). Since IKK2 phosphorylation of I κ B α is dependent on complexing

with the NEMO subunit, this data provides evidence supporting the hypothesis that CK2 may directly regulate IKK2 activity for phosphorylation of I κ B α in UM-SCC-6 cells.

Discussion

We previously discovered that NF- κ B is aberrantly activated and promotes tumorigenesis in human HNSCC and murine SCC, (4–8,10). Nuclear NF- κ B activation has been broadly demonstrated and associated with progression in intraepithelial pre-malignant and malignant squamous neoplasms of the head and neck as well as uterine cervix (9,33). We have shown that expression of an S32A/S36A I κ B α mutant unresponsive to IKK phosphorylation strongly inhibited NF- κ B activation in HNSCC and murine SCC (7,10). Tamatani et al detected increased IKK-mediated phosphorylation of I κ B α and NF- κ B activation in 3 HNSCC cell lines relative to that observed in 5 primary gingival keratinocyte cultures, indicating that IKK and NF- κ B are aberrantly activated together in HNSCC (34). Gapany et al showed that cytosolic CK2 expression and activity are also increased in HNSCC tumor specimens and cell lines (19). In the present study, we directly examined the hypothesis that CK2 promotes IKK mediated aberrant activation of NF- κ B in HNSCC. NF- κ B activation was specifically inhibited by siRNA targeting the β subunit of CK2, and kinase dead mutants of the IKK1 and IKK2 subunits, implicating both the alternative and classical IKK pathways in NF- κ B activation. We found that CK2 contributes to the activation of IKK and NF- κ B in response to serum factor(s). rCK2 α was shown to phosphorylate rIKK2, as well as to promote immunoprecipitated IKK complex from HNSCC to phosphorylate the N-terminal S32/S36 of I κ B α . We conclude that the aberrant NF- κ B activity in HNSCC cells in response to serum is partially through a novel mechanism involving CK2 mediated activation of IKK2, making these kinases candidates for selective therapy to target the NF- κ B pathway in HNSCC. This is the first study to identify a potential mechanism linking the independent clinical pathologic observations that CK2 is an upstream regulator of IKK and NF- κ B activation in HNSCC.

Several observations from this and other studies in our laboratory suggested an alternative mechanism of activation and role of IKK in NF- κ B activation in HNSCC. First, we observed that both IKK1 and IKK2 contribute to NF- κ B activation. The inhibition of NF- κ B reporter activity in different UM-SCC cell lines by specific inhibition of either IKK2 with kinase dead IKK2^{KA}, IKK2 siRNA or IKK2 inhibitor PS-1145, or by that of IKK1 with IKK1^{KA} or IKK1 siRNA along were significant, reproducible, but incomplete. While IKK2 has been shown to mediate activation of NF- κ B1/Rel A (p50/p65), IKK1 has been reported to promote processing of p100 to p52 (NF- κ B2), an alternative NF- κ B activation pathway (29). Consistent with this, we found evidence for higher expression levels of endogenous NF- κ B inducible genes I κ B α and NF- κ B2 (data not shown), increased degradation rate of I κ Bs, and processing of p100 following cycloheximide treatment in UM-SCC-6 cells (data not shown). Furthermore, the IKK2^{AA} mutant did not inhibit NF- κ B activation, and the IKK1^{AA} mutant enhanced activation of NF- κ B activity. These results suggested that the signaling mediated by these IKK subunits in HNSCC may result from signal(s) and/or mechanism(s) that are distinct from those mediating classical activation of IKK and NF- κ B (28).

CK2 is a highly conserved pleiotropic and ubiquitous serine and threonine kinase with a wide range of substrates involved in carcinogenesis and tumor progression. More than 300 CK2 substrates have been identified (13,35), the majority of which are proteins that are involved in transcription, cell cycle regulation, cell proliferation, cell survival, gene expression and signal transduction. CK2 phosphorylation has been shown to inhibit apoptosis, and favor cell proliferation and oncogenic transformation (13,36). In this study, we provide evidence that CK2 is a key mediator of overall NF- κ B activation, and functions to enhance IKK kinase phosphorylation of I κ B α . The CK2 inhibitor apigenin or specific siRNA targeting CK2 β were sufficient to inhibit NF- κ B activity, indicating that the over-expression of CK2 and increased

CK2 activity found in prior studies (19,20) may be responsible for mediating the aberrant activation of NF- κ B in HNSCC.

CK2 has previously been shown to phosphorylate multiple sites in the C-terminal PEST domain of I κ B α , including the response involved in ultraviolet (UV) light-induced NF- κ B activation (14,16). CK2 has also been reported to phosphorylate S529 of the RelA/p65 subunit (17,18). In addition to these targets, we have found that catalytically active recombinant CK2 α (rCK2 α) can phosphorylate both recombinant IKK2 (rIKK2) or IKK1 (rIKK1, data not shown) *in vitro*, and that incubation of IKK complex with rCK2 α resulted in increased IKK2 kinase activity for the phosphorylation of S32/S36 N-terminus of I κ B α , a novel and distinct function from that of CK2 as a known C-terminal PEST domain I κ B kinase. This finding is also supported by protein sequence analysis of IKK1 and 2, which reveals multiple CK2 phosphorylation motifs. Additionally, the decreased IKK kinase activity in UM-SCC-9 and SFM cultured UM-SCC-6 cells were associated with lower expression levels of CK2 β in these cells, adding another line of evidence that CK2 is an upstream regulator of IKK and NF- κ B activation.

CK2 has been linked to aberrant NF- κ B activity in cancer cells derived from human breast, hepatic, and colon carcinomas. (30,37–41). However the relative contribution of CK2 to NF- κ B activation appears to vary among different cancers. Compared to CK2 activation of NF- κ B in other type of cancer cells (30,37–41), our CK2 β siRNA data indicates a dominant effect of CK2 as a holo-enzyme on NF- κ B activation in UM-SCC cells. On the other hand, our data does not rule out the possibility of direct effects of CK2 on activating the NF- κ B pathway at other levels, as multiple CK2 phosphorylation motifs have been identified in every member of the NF- κ B and I κ B families. Thus, the presence of CK2 target sites in I κ B and p65 as well as the potential sites in IKK and other NF- κ B and I κ B family members could explain why we observed nearly complete inhibition of NF- κ B activity by blocking CK2 activity. More detailed characterization of the molecular basis of the interaction between CK2 and IKK is needed, and may lead to additional specific targets for cancer treatment.

An important finding of the present study is the demonstration that CK2 and IKK mediate the altered activation of NF- κ B in response to serum factor(s). The increase in CK2 and NF- κ B activation in the majority of HNSCC specimens relative to normal mucosa (9,20) indicate that the response of these pathways to serum factor(s) is altered and precedes culture, rather than being a mere consequence of serum induction in culture. Indeed, we have shown that the increased NF- κ B activity in murine SCC cells occurs with tumor progression *in vivo*, and favors tumorigenesis and metastasis in the host environment (5). Consistent with this, the increased nuclear localization of NF- κ B observed in human squamous dysplasias has been shown to be associated with higher risk of progression in a recent clinical pathologic study (9).

The factor(s) in serum and/or the host environment that contribute to induction of CK2, IKK and NF- κ B remain to be elucidated, and may provide additional targets for therapy. Autocrine factors produced by HNSCC (42,43) or paracrine factors produced by tumor stromal fibroblasts (44) have been shown to enhance activation of NF- κ B in HNSCC. A number of components contained in serum, including cytokines, growth factors, different types of albumins, zinc, copper, and free thiols, have been reported to affect NF- κ B activity (13,24–26,31,43–49). Since the serum factors contributing to NF- κ B activation in UM-SCC were heat sensitive, labile protein factors seem the most likely candidates.

We have previously evaluated the growth factor EGF and cytokine IL-1 as possible candidates (42,43), since they are factors that are known to activate NF- κ B in non-malignant and malignant cells. Although NF- κ B was found to be inducible by EGF, C225, the specific antagonist of anti-EGFR antibody, inhibited only inducible, but not aberrant NF- κ B activity, making the

EGFR receptor an unlikely candidate for the aberrant NF- κ B activation in HNSCC (42). We have previously obtained evidence that the IL-1/IL-1Receptor (IL-1R) pathway may be one of the stimuli contributing to the aberrant activation of NF- κ B in HNSCC. We found that transient expression of an intracellular form of the IL-1Receptor Antagonist (IL-1RA) could significantly inhibit NF- κ B reporter activity and cytokine IL-8 gene expression in UM-SCC-9 and 11B cell lines (43). Recently, interruption of the IL-1 signal pathway by expression of siRNA knocking down expression of IL-1R1, or a dominant negative mutant of the essential Toll receptor linker MyD88, was found to strongly inhibit NF- κ B activation in 5/6 UM-SCC lines, including the UM-SCC-6, 9 and 11A and B cell lines (L. Bagain et al, unpublished observations).

Interestingly, a recent study in malignant keratinocyte lines suggests that the IL-1 pathway and intracellular IL-1R Antagonist may play a role in regulating signal degradation of I κ B α and other transcription factors by the COP9 (CSN) signalosome that includes CK2 (50). Such an alternative pathway for activation of NF- κ B by IL-1 and possibly other factors could explain the enhancement of NF- κ B reporter activity by IKK1^{AA} and lack of inhibitory effect we observed with IKK2^{AA}, both of which are deficient in the phosphoacceptor sites mediating classical activation of NF- κ B. It would be interesting if this signalosome containing CK2 interacts with the IKK signalosome and other NF- κ B pathway components. Dissection of the role of CK2 or other intermediate kinases as possible oncogenes mediating aberrant activation of IKK and NF- κ B in response to exogenous factors in HNSCC may also be important to development of therapy.

In summary, we found that CK2 contributes to the activation of IKK and NF- κ B in response to serum factor(s), which suggests that CK2 and IKK2 are key candidates for targeting the NF- κ B pathway in HNSCC. This is the first study to identify a potential mechanism linking the independent clinical pathologic observations that CK2 and NF- κ B are activated and associated with decreased prognosis in HNSCC. Further clinical and molecular studies are indicated to confirm the role and further elucidate the factor(s) and mechanisms involved in CK2 activation of IKK and NF- κ B in HNSCC.

Acknowledgements

Grant support: NIDCD Intramural Research Project Z01-DC-00016 (C. Van Waes), and Howard Hughes Medical Research Institute-NIH Scholars Program (J Yeh). Critical review and comments of Keith Brown of the National Institute of Allergy and Infectious Disease, and David Gius of the National Cancer Institute are appreciated.

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Fig. 1. NF-κB is aberrantly activated in UM-SCC cells

A. Electromobility shift assay for NF-κB DNA binding activity. HEKa and the UM-SCC cell lines indicated in the figure were cultured as described in methods. 5 μg of nuclear extract protein from each cell line was used for binding to radiolabeled oligonucleotide containing the NF-κB-response element. A variable increase in NF-κB-response element DNA binding activity was detected in most UM-SCC cells, when compared to HEKa cells. Free probe was used as negative control. Wild type and mutant cold oligonucleotide were incubated with nuclear extracts from UM-SCC-5 as an NF-κB response element specific binding control. Oct as a control for nuclear extract integrity and loading is shown in overlay. **B. NF-κB Reporter Activity from UM-SCC Cells.** NF-κB reporter activity was measured by co-transfecting cells with pNF-κB-*cis*-luciferase reporter plasmid containing a 5x NF-κB enhancer consensus element. NF-κB reporter activities that are presented here represent an average of multiple measurements that were normalized as a percentage of activity of UM-SCC-6 cells. Cell lines shown here include UM-SCC1, 6, 9, 11A, and 11B as indicated. The transfection conditions were optimized to reach over 90% transfection efficiency for each cell line as determined by β-Galactosidase gene under control of a constitutive CMV promoter (bottom panel). Variable

NF- κ B reporter activities were observed. UM-SCC-6 cells showed significantly high NF- κ B reporter activity comparing to other cell lines ($p < 0.01$) while UM-SCC-9 cells showed the significantly low NF- κ B reporter activity comparing to other cells ($p < 0.01$). **C. I κ B α expression and Degradation Time Course From UM-SCC-6 and -9 Cells Following Cycloheximide Treatment.** Cells were treated with 50 μ g/ml cycloheximide for 0.5 to 4 hours as indicated. The protein levels of I κ B α were detected by immunoblot analysis and normalized to total protein detected from the membrane. • ♦ UM-SCC-6, UM-SCC-9 cells. Data show that the I κ B α protein level in UM-SCC-6 cells is 1.8 fold of that in UM-SCC-9 cells and that the half life of I κ B α in UM-SCC-6 cells is 1.5 hr, half of that in UM-SCC-9 cells (3 hours). **D. Comparison of IKK Kinase Activity Between UM-SCC-6 And -9 Cells.** Cell lysates from UM-SCC-6 and -9 cells were immunoprecipitated by NEMO. IKK kinase activity was measured as incorporated [³³P]-I κ B α by using an N-terminal I κ B α -GST fusion protein as substrate (WT). The result is normalized by total I κ B α and, then, IKK2 protein detected from the same membrane. A mutant S32G/S36A N-terminal I κ B α -GST fusion protein was used as negative control (Mut). Data show that IKK kinase activity in UM-SCC-6 (SCC-6) cells is one fold higher than that in UM-SCC-9 (SCC-9) cells. The detected gel bands and corresponding measurements of the intensities of these bands are shown under the chart and in the table respectively.

Fig. 2. Effects of IKK mutants on NF-κB Reporter Activity

UM-SCC-6 Cells were cotransfected with pNF-κB-luciferase plasmid and one of the pcDNA3-IKK vectors (IKK1-AA=S176A/S180A; IKK1-EE=S176E/S180E; IKK1-KA=K44A, IKK1-WT=wild type; IKK2-AA=S177A/S181A; IKK2-EE=S177E/S181E; IKK2-KA=K44A, IKK2-WT=wild type), as indicated, for 24 hours. pCK is used as a negative control. NF-κB activity is normalized to pCK. A, Effects of IKK2 on NF-κB reporter activity; B, Effects of IKK1 on NF-κB reporter activity. Both wild type (IKK1-WT, IKK2-WT) and dominant positive (IKK1-EE, IKK2-EE) of IKK1 and IKK2 enhanced the NF-κB reporter activity (IKK1-WT to untreated control $p < 0.05$, IKK1-EE, IKK2-WT and IKK2-EE to their corresponding untreated control $p < 0.001$). Kinase dead IKK2-KA inhibited ~65% of the

constitutive NF- κ B reporter activity ($p < 0.001$). Dominant negative signal mutant IKK2-AA showed no inhibitory effect on NF- κ B activity ($p > 0.10$). Kinase dead IKK1-KA inhibited ~50% of NF- κ B activity ($p < 0.001$). Little inhibitory but enhancing effect was observed from kinase dead IKK1-AA ($p < 0.05$).

Fig. 3. CK2 is responsible for the Aberrant NF- κ B activity from UM-SCC cells

A. Apigenin inhibits Aberrant NF- κ B reporter activity from UM-SCC cells. UM-SCC-6, 9, 11A and 11B cells, as indicated in the figure, were exposed to 100 μ M Apigenin for 2 hours prior to the DNA transfection for NF- κ B luciferase reporter assay. The reporter activities from each cell lines were normalized to their corresponding untreated-control cells (open column, Control; solid column, Apigenin). The data showed that Apigenin inhibited ~90% of the NF- κ B reporter activity from UM-SCC-6 cells ($p < 0.005$) and 70% of that from UM-SCC-9 cells ($p < 0.05$). 90% and 80% reduction of NF- κ B reporter activities were also observed from UM-SCC-11A ($p < 0.005$) and UM-SCC-11B cells ($p < 0.05$). **B and C, CK2 β siRNAs knocks down mRNA levels in UM-SCC-6 and UM-SCC-9 cells.** UM-SCC-6 and -9 cells were transfected with CSNK2B (CK2 β), scrambled, or a universal control siRNAs for 24, 48 and 72 hours as indicated. Untreated, no siRNA transfection; Ctrl-Universal, Universal negative control siRNA from Ambion; Ctrl-Scramble, Stealth scrambled siRNA control for CK2 β siRNA; CSNK2B, siRNA specially targeting CK2 β mRNA. Data show a }time dependent decrease of CK2 β mRNA levels from both cell lines (UM-SCC-6, $p < 10^{-9}$; UM-SCC-9 cells, $p < 10^{-5}$). **D and E. CK2 β siRNA inhibits the Aberrant NF- κ B activity in UM-SCC cells.** Cells were transfected with siRNAs targeting CK2 β subunits as well as control siRNAs, as indicated, for 48 hours prior to the transfection for NF- κ B luciferase reporter assay. **D.** The NF- κ B reporter activities

from UM-SCC-6 cells; E. The NF- κ B reporter activities from UM-SCC-9 cells. CK2 β siRNA significantly reduces the NF- κ B reporter activity (90%) from UM-SCC-6 cells ($p < 0.05$). ~60% reduction of the NF- κ B reporter activity was observed from UM-SCC-9 cells, but this change is not statistically significant. **F. CK2 β siRNA inhibits expression of endogenous NF- κ B inducible gene I κ B α and NF κ B2/p100 in UM-SCC cells.** Messenger RNA was isolated from cell lysates of untreated UM-SCC-6 cells (open bar) and cells transfected with siRNAs targeting CK2 β subunits (slashed bar) as well as the scramble control siRNA (solid bar) after 48 hours. A Quantigene kit was used for mRNA quantification of I κ B α and NF κ B2/p100 as indicated. Data were normalized to its corresponding 18S rRNA level as detected at the same time. Data show statistically significant decrease of I κ B α ($p < 10^{-6}$) and NF κ B2/p100 ($p < 10^{-7}$) in CK2 β siRNA transfected cells compared to the corresponding scramble control.

Fig. 4. CK2 and IKK mediate NF-κB response to serum in UM-SCC cells

A. Aberrant NF-κB reporter activity is dependent on heat sensitive components of FBS
UM-SCC-6, 9, 11A and 11B cells, as indicated in the figure, were either cultured in MEM with 10% non-heat inactivated FBS (FBS-NHI ■), 10% heat inactivated FBS (FBS-HI ■), switched to supplemented serum free MEM medium (SFM-new), or adapted to SFM (SFM-Adv ■) prior to the transfection for NF-κB reporter activity. The NF-κB reporter activity levels were normalized to the level of their corresponding FBS-NHI. ANOVA analysis and Student-Newman-Keuls analysis comparing each of the treatment groups is shown. A reduction of NF-κB reporter activity was observed from the cells that were cultured under both SFM-new and FBS-HI conditions when compared to that of the cells under FBS-NHI conditions. Adaptation to SFM further decreased NF-κB reporter activity by 95% in UM-SCC-6, 11A and 11B cells and 83% in UM-SCC-9 cells ($p < 0.01$). The level of NF-κB reporter activity from SFM-adv is significantly lower than that of both SFM-new and FBS-HI ($p < 0.01$). No statistically significant difference was detected between SFM-new and FBS-HI. **B. Comparison of CK2**

kinase activity in cells that were cultured in SFM or FBS. Total cell lysates from UM-SCC-6 cells that were cultured either in SFM (SFM) or FBS (FBS) were used for the CK2 kinase assay. A recombinant active CK2 (Active CK2) was used as positive control. 100 μ M Apigenin was applied to separate reactions in parallel to each of the conditions (solid, Apigenin). CK2 activity (open, Ctrl) from the cells that were cultured in SFM was reduced to about 50% of that in FBS ($p < 0.001$). The cell lysates that were treated with apigenin (Apigenin) brought down the corresponding CK2 activity from each of these lysates by ~75% of that FBS and ~50% of that of SFM ($p < 0.05$). **C. Comparison of CK2 β expression level in cells.** UM-SCC-6 cells grown in FBS-NHI or SFM-adp and UM-SCC-9 cells grown in FBS-NHI, as indicated, were compared for their CK2 β mRNA levels. CK2 β mRNA expression levels were measured as described in Fig 3 F using Quantigene kit method. Data show a statistically significant decrease of CK2 β mRNA in both SFM treated UM-SCC-6 cells and UM-SCC-9 cells compared to FBS control UM-SCC-6 cells ($p < 0.01$ for SFM and $p < 0.001$ for UM-SCC-9 Cells). **D. Comparison of IKK kinase activity from the cells that were cultured in SFM or FBS.** UM-SCC-6 cells were cultured in SFM or FBS. IKK complexes were IPed by an anti-NEMO antibody from 200 μ g total protein of whole cell lysate of each culture. IKK kinase activity was measured with an N-terminal 72 amino acid I κ B α -GST fusion protein as substrate and a S32G/S36A mutant of the I κ B α -GST fusion protein as negative control. Quantified total IKK kinase activity was presented as a ratio of incorporated 33 -P radio-activity of phosphorylated- I κ B α -GST quantified by autoradiography to the quantified total-I κ B α -GST protein level on the same gel for total IKK kinase activity, and further normalized to the total protein levels of IKK2} on the same gel. A 60% reduction of the normalized IKK kinase activity from these cells were observed. A recombinant active IKK2 was used as positive control (data not shown). . The detected gel bands and corresponding measurements of the intensities of these bands are shown under the bar graph and in the table respectively.

Fig 5. CK2 Facilitates IKK Kinase Activity

A. IKK2 Is a Substrate of CK2. rIKK2 was incubated without (–) or with 0.5 (0.5) and 5ng (5) of rCK2α and 5ng of rCK2α along with 40μM apigenin (5+Apigenin), as indicated, in the presence of [³³]P-GTP followed by SDS-PAGE gel separation, transfer, autoradiography and immunoblot analysis for detection of phosphorylated IKK2 and protein levels of IKK2 and CK2α. CK2 phosphorylation of IKK2 activity is presented as the radiation activity detected by autoradiography and normalized to the protein levels of IKK2 detected from the membrane. Data show CK2α dose dependent phosphorylation of IKK2 and complete inhibition of this effect by apigenin. **B. CK2 Promotes IKK Kinase Activity** Catalytically active rCK2α (0.5ng (CK2 0.5ng) or 5ng(CK2 5ng)) and 5ng of rCK2α along with 40μM apigenin (CK2 5ng +Apigenin) respectively were incubated with IKK complex that was immunol precipitated from UM-SCC-6 cell lysate. rCK2α was, then, washed out prior to IKK kinase assay. A fraction of untreated IKK complex was used as negative control (Ctrl). IKK kinase activity was measured with the same substrate as in Fig 4D. Quantified total IKK kinase activity was presented as described in Fig 4D. An 13% and 60% increase of total IKK kinase activities from IKK complex preincubated with 0.5ng and 5ng rCK2α respectively was observed compared to untreated control. 40μM anpigenin completely inhibit the rCK2α induced IKK kinase activity. The detected gel bands and corresponding measurements of the intensities of these bands are shown under the chart and in the table respectively.