Involvement of Two NF-κB Binding Elements in Tumor Necrosis Factor α-, CD40-, and Epstein-Barr Virus Latent Membrane Protein 1-mediated Induction of the Cellular Inhibitor of Apoptosis Protein 2 Gene*

Received for publication, February 14, 2000, and in revised form, April 3, 2000
Published, JBC Papers in Press, April 6, 2000, DOI 10.1074/jbc.M001202220

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The antiapoptotic function of NF-κB is believed to be mediated through the induction of antiapoptotic genes. Among the antiapoptotic genes, cellular inhibitor of apoptosis protein 2 (c-IAP2/HIAP-1/MIHC) is originally identified as a molecule recruited to the tumor necrosis factor (TNF) receptor complex, and its expression is preferentially up-regulated by TNF and other stimuli activating NF-κB. However, direct evidence of transcriptional regulation of NF-κB on the c-IAP2 gene is still missing. Here, we have cloned and characterized the promoter region required for NF-κB-dependent transcription of the c-IAP2 gene. Sequencing of a 3.5-kilobase fragment of the 5′-flanking region of the c-IAP2 gene has identified a TATA-like sequence and potential binding sites for nuclear factor of activated T cells, interferon regulatory factor 1, activator protein 1, glucocorticoid response element, and three putative NF-κB binding elements. Deletion and mutational analysis of the 5′-flanking region linked to the luciferase gene revealed that transcriptional activation by TNF or interleukin 1 is mediated cooperatively by two NF-κB binding sites. Electrophoretic mobility shift assays characterized that the two NF-κB sites can be recognized and bound by the NF-κB p50/p65 heterodimer. In addition, the transcription of c-IAP2 promoter was strongly up-regulated when CD40 or Epstein-Barr virus latent membrane protein 1 was overexpressed.

Stimulation of cells with tumor necrosis factor α (TNF) generates two seemingly conflicting signals; one that triggers apoptotic cell death and the other that antagonizes the apoptotic signal by activating transcription factor NF-κB (1). The overall outcome in a specific cell type is dependent on the balance of these two signals. In cells resistant to TNF-induced apoptosis, inhibition of NF-κB activation attenuates apoptosis resistance (2). Furthermore, NF-κB subunit p65-disrupted cells are more sensitive to TNF-induced apoptosis (3). The protective role of NF-κB against apoptosis is believed to be mediated by the induction of various antiapoptotic genes, including IEX-1L (4), Bcl-2 and Bcl-x (5), A20 (6), and some members of the inhibitor of apoptosis (IAP) family such as XIAP (7), c-IAP-1, and c-IAP2 (8–10). Among these, c-IAP2 (cellular inhibitor of apoptosis protein 2), also known as HIAP-1 (11) and MIHC (12), was initially identified as a molecule that is recruited to the TNF receptor via its association with the TNF receptor-associated factors, TRAF1 and TRAF2 (13). TRAF molecules are proposed to function as adapter proteins conveying the TNF receptor-mediated signals (14). It has been suggested that c-IAP2 can inhibit apoptosis by modulating the TNF-induced NF-κB activity (10). It was observed that, in Jurkat T cells, TNF treatment led to NF-κB-dependent induction of c-IAP2 gene, and conversely, c-IAP2 was able to activate NF-κB via an IκBα-targeting mechanism; this consequently suppressed TNF-induced apoptosis. Later studies showed that c-IAP2 exhibits its antiapoptotic function by directly binding and inhibiting downstream cell death protease caspases-3, -7, and -9, but not upstream protease caspase-8, which is implicated in TNF-induced cell death signaling (15, 16). However, a recent study suggests that c-IAP2 requires TRAF1, TRAF2, and c-IAP1 activities for the full suppression of TNF-induced cell death at the level of caspase-8, all of which are transcriptionally activated by NF-κB (17). In cells in which NF-κB activation was prevented with dominant negative IκBα, ectopic expression of c-IAP-1, c-IAP2, TRAF1 and TRAF2 together fully suppressed TNF-induced apoptosis and caspase-8 activation, substituting for the antiapoptotic effect of NF-κB. In the same cells, however, either c-IAP1 or c-IAP2 alone was sufficient to suppress etoposide-induced cell death by direct inhibition of caspase-3 (17). These results support the hypothesis that NF-κB activates a group of gene products that function cooperatively to suppress TNF-induced apoptosis by inhibiting the activation of upstream death protease caspase-8.

In contrast to the mode of action of c-IAP2, little is known about how c-IAP2 is regulated, except that it is under the control of NF-κB. Transcription of c-IAP2 gene was found to be also up-regulated by treating endothelial cells with interleukin 1 (IL-1) or lipopolysaccharide (18) and by CD40 stimulation on B lymphocytes (19), which are stimuli that lead to NF-κB

* This work was supported by Genetic Engineering Grant 1998-019-F00041 from the Ministry of Education and by Korea Science and Engineering Foundation Grant 1999-0403-06-01-3. The costs of publication of this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF233684. § To whom correspondence should be addressed: Dept. of Biology, College of Science, Yonsei University, Seoul 120-749, South Korea. Tel.: 82-2-361-4084; Fax: 82-2-312-2242; E-mail: thlee@yonsei.ac.kr.

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1 The abbreviations used are: TNF, tumor necrosis factor α; c-IAP, cellular inhibitor of apoptosis protein; TRAF, TNF receptor-associated factor; IL-1, interleukin 1; EMSA, electrophoretic mobility shift assay; EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; AP-1, activator protein 1; NFAT, nuclear factor of activated T cell; LUC, luciferase; TNFR1, p55 TNF receptor; NIK, NF-κB-inducing kinase; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; TCR, T cell receptor.
activation. It has been demonstrated that the transcriptional activation of other antiapoptotic genes such as A20, Bcl-X, and Bfl1/A1 by NF-κB is through one or two NF-κB binding sites resided in their promoter regions (5, 20, 21). It remains to be elucidated, however, whether a similar transcription-regulating mechanism is involved in c-IAP2 gene expression. Although the partial 5′-flanking region surrounding the putative transcription start site of c-IAP2 was reported (22), the sequence information was not enough to gain an understanding of the regulation of c-IAP2 gene expression. In the present study, we sequenced the 5′-flanking region (3.5 kilobases (kb)) of the c-IAP2 gene. Deletion analysis revealed that the region conferring inducibility by TNF or IL-1 is localized downstream from the previously proposed transcription start site and contains three putative consensus NF-κB binding sites and other potential transcription factor binding elements. Electrophoretic mobility shift assays (EMSA) and site-directed mutagenesis analysis of the NF-κB binding sites demonstrated that two NF-κB elements are required for promoter activity and that they function cooperatively in mediating TNF-induced c-IAP2 promoter activation. Moreover, we showed that the c-IAP2 promoter activity is strongly enhanced in cells transfected with expression plasmids for CD40 and Epstein-Barr virus (EBV) oncoprotein latent membrane protein 1 (LMP1).

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Human embryonic kidney 293 cells and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). Recombinant human TNF was kindly supplied by Dr. H. H. Chung of LG Biotech Research Institute, Daejeon, Korea. Recombinant IL-1β was purchased from Genzyme. Poly(I:dC) was obtained from Roche Molecular Biochemicals. All other reagents were purchased from Sigma if not otherwise stated. Expression plasmid for LMP1 was obtained from Dr. W. G. Lee (Myung-Ji University, Yongin, Korea).

Isolation of Genomic Clones of the c-IAP2 Gene and Sequencing of Its 5′-Flanking Promoter Region—A genomic library from HeLa cells was screened with the full length human c-IAP2 cDNA. A total of 8 × 10^6 plaques were screened, and nine positives were isolated and further characterized by restriction mapping and Southern blot analysis. The Spel restriction fragment of c-IAP2 promoter was isolated and cloned into pBluecript KS(−) (Stratagene) as a potential genomic fragment harboring promoter region extending from the 5′-untranslated region of the c-IAP2 gene. Further analysis revealed that a unique Spol site within the 8.0-kb Spel fragment represents the restriction site embedded closest to the 5′-end of the longest cDNA sequence filed (GenBankTM accession number AF070674). The 3.5-kb fragment upstream of the Spol was isolated after restriction with the pBluescript multicloning site-derived Xhol and Spol and cloned to the respective sites of pSP73 (Promega), resulting in pSP73-3.5kbSh. Nucleotide sequences of the insert on both strands were determined by the use of automatic DNA sequence.

Preparation of 5′-Deleted Constructs of the c-IAP2 Promoter with Luciferase Reporter Gene—A series of plasmids containing various sizes of the 5′-flanking region of c-IAP2 promoter were constructed by inserting DNA fragments into the basic luciferase reporter plasmid, pGL2 (Promega). The 3.5-kb SpeI-HindIII fragment from pSP73-3.5kbSh was cloned into the NheI and HindIII sites of pGL2, generating pGL2(-3.5kLUCC). The 2.1-kb BamHI-HindIII fragments were inserted, respectively, into the NheI/BgII and BglII/HindIII-digested pGL2, resulting in pGL2(-3.5kLUCC) and pGL2(-1.4kLUC). For a fine directional deletion cloning within the 1.4-kb BamHI-HindIII fragment, DNA fragments were amplified using polymerase chain reaction with various forward primers and a common reverse primer complementary to the 3′ end of the plasmid and is a common reverse primer primer complementary to the 3′ promoter sequence that is embedded downstream of the multicloning sites of pSP73. Following are the synthetic forward primers incorporating a Xhol site at their 5′-end: 5′-ccgctagCTTTTCCTGGCCACCC-3′ (−200 to −153), 5′-ccgctagCTTTTCCTGGCCACCC-3′ (−147 to −124), 5′-ccgctagCTTTTCCTGGCCACCC-3′ (−100 to −184), 5′-ccgctagCTTTTCCTGGCCACCC-3′ (−147 to −124), and 5′-ccgctagCTTTTCCTGGCCACCC-3′ (−100 to −184).

RESULTS

Cloning and Identification of the c-IAP2 Promoter Region Conferring NF-κB-dependent Activation—For cloning of the 5′-flanking region of the c-IAP2 gene, a λASH II HeLa cell genomic library was screened with a probe directed against the full length c-IAP2 cDNA sequence. Southern blot and restriction enzyme analysis of DNA from nine strongly hybridizing clones led to the identification of one clone termed λ10-a, which contained the 5′-flanking region of c-IAP2. After digestion with various restriction enzymes, fragments of the genomic insert were cloned into pBluescript KS(−) and their partial nucleotide sequences were determined. Fig. 1A shows the map of clone λ10-a relative to the genomic organization reported by Young et al. (22). Because it has been reported that c-IAP2 gene is induced by TNF (10, 17), we initially tried to examine whether the region upstream from the 5′-end of the longest cDNA sequence (GenBankTM accession number AF070674) possesses the promoter activity conferring TNF inducibility by reporter gene transfection analysis. The 3.5-kb SpeI-SphiI fragment and its 5′- or 3′-deleted fragments were linked to the luciferase reporter gene in the pGL2 basic plasmid (Fig. 1B), and the resultant reporter plasmid construct was transiently transfected into human embryonic kidney cell line 293 cells. After 24 h, transfected cells were treated with TNF for 4 h and examined for TNF-induced luciferase expression. As shown in Fig. 1C, the SpeI-SphiI fragment showed a strong promoter activity, enhancing the luciferase activity by 5-fold upon TNF stimulation. The promoter activity of the 5′-flanking region was not changed with the transfection of the reporter plasmids having the BamHI-SphiI segment in which the 2.1-kb region was removed from the 5′-side of the SpeI-SphiI fragment, yielding a 10-fold induction. However, the SpeI-BamHI segment upstream of the putative transcription start site, determined previously using the 5′-rapid amplification of cDNA ends pGL2.

Transfection and Luciferase Assays—Transfection was carried out by the CaPO4-DNA precipitation method using N,N-bis[2-(hydroxymethyl)-2-aminoethanesulfonic acid (BES) buffer as described elsewhere. 2 μg of each reporter luciferase constructs was transfected along with 0.5 μg of pCDM8-β-galactosidase (22) when the cells (seeded on 96-well plates) reached 70% confluence. The 70% post-transfection, TNF or IL-1β was added for 4 h as indicated. Transfectants were lysed in 0.15 ml of lysis buffer (Promega) and centrifuged at 10,000 × g for 5 min to remove cell debris. The resulting clear lysates were assayed for luciferase and β-galactosidase activity, and the values of the luciferase assay were normalized with respect to the values of the β-galactosidase assay for each transfection.

Preparation of Nuclear Extracts and EMSA—293 and HeLa cells were treated with TNF at the indicated times. Nuclear extracts were prepared as described by Dignam et al. (24), quantitated by the Bradford assay (Bio-Rad), and stored at −70 °C. The oligonucleotide probes for EMSA corresponded to the three potential NF-κB binding sites in the c-IAP2 promoter (NF-κB site 1: sense, 5′-ATGGAATCCCTCCGA-3′ and antisense, 5′-TCGGGGATTTCCTC-3′; NF-κB site 2: sense, 5′-AG-TGGTTGGCCAGG-3′ and antisense, 5′-CGTGGAAACCCACT-3′; NF-κB site 3: sense, 5′-GCTGGAGTTCCCTC-3′ and antisense, 5′-AG-GGAAACTCCAGC-3′). Two oligonucleotides complementary to each other were annealed to generate a double stranded probe. End labeling was accomplished by treatment of T4 polynucleotide kinase in the presence of 32P-ATP. Approximately 1 μg of the labeled probe was mixed with 2.4 μg of a nuclear protein in a total of 20 μl of the binding buffer (20 mM HEKES, pH 7.9, 60 mM KCl, 1 mM MgCl2, 20 μg EDTA, 0.5 mM dithiothreitol, 10% glycerol) containing 2 μg of poly(dI:dC). After incubation for 20 min, the reaction mixture was separated on a 6% non-denaturing polyacrylamide gel with 0.5% TBE buffer (40 μM Tris borate, 1 mM EDTA, pH 8.0). The gel was vacuum-dried and subjected to autoradiography. For competition experiments, a 100-fold molar excess of unlabeled double stranded probe was added prior to the addition of the labeled probe as specified. For supershift experiments, 0.2 μg of antibody was added to the mixture before the addition of the labeled probe. All antibodies used in these experiments were purchased from Santa Cruz Biotechnology.
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Fig. 1. Structural organization of the 5'-flanking region of the c-IAP2 gene and localization of its promoter region imparting TNF inducibility. A, partial restriction map of human the c-IAP2 clone 170-a is depicted relative to the published 5'-flanking genomic organization of the c-IAP2 gene (22). Numbers in parenthesis of the restriction sites represent the approximate length in kilobases from the Sall site located immediately downstream of the ΔDASH II left arm. B, three different luciferase reporter plasmids were constructed to localize the promoter region conferring NF-κB activation-dependent c-IAP2 gene induction. C, luciferase assays were carried out with extracts of 293 cells transfected with 2 μg of each reporter plasmid for 24 h and then treated with TNF (20 ng/ml) during the last 4 h of the transfection period or left untreated. D, each deletion construct was cotransfected with 0.5 μg of the expression plasmids, NIK or p65. 24 h after transfection, 293 cell lysates were prepared and processed for luciferase assays. Relative luciferase activity was expressed as a percentage of the value obtained from cells transfected with the NIK expression plasmid. The results shown are representative of three different experiments.

DNA Sequence Analysis of the c-IAP2 Promoter Region Imparting Inducibility by TNF Reveals Putative Cis-regulatory NF-κB Elements—The 3.5-kb SpeI-SphI segment of the 5'-flanking region of the c-IAP2 gene was sequenced. The resulting sequence features a number of elements characteristic of eukaryotic promoters and their regulatory regions (Fig. 2). Several attempts were made but failed to assign the transcription start site by primer extension analysis due to the inherent technical difficulty of obtaining the intact mRNA whose length of the 5'-untranslated region of c-IAP2 mRNA is estimated to be longer than 3.5 kb. For convenience, however, we arbitrarily assigned the sequence number +1 for the nucleotide G that was documented as a 5'-end residue of the longest cDNA clone. Based on this numbering, a TATA-like box (TTTAAA) was identified at position −42. Several potential regulatory elements were found by computer search using the MatInspector software (Genomatrix). Two interferon regulatory factor-1 consensus sequences were detected at positions −130 and −475. Two potential binding sites for activator protein-1 (AP-1) (at −220 and −294) and four nuclear factor of activated T cells (NFAT) binding sites (at −301, −354, −821, and −1086) as well as a glucocorticoid responsible element (at −514) were found. Most importantly, three putative NF-κB binding sites were identified at positions −147, −197, and −210.

Analysis of Deletion Constructs of c-IAP2 Promoter—To localize important regulatory regions within the 5'-flanking sequence of c-IAP2, which confer TNF or IL-1 inducibility, 293 or HeLa cells were transfected with constructs of serially deleted sequences of the c-IAP2 promoter linked to the luciferase gene. The length of the 5'-flanking region incorporated into the constructs ranged from 93 base pairs to 1.4 kb (Fig. 3A). Upon transfection of the cells with the eight different constructs, cultures were divided into three groups that were, respectively, left untreated, treated with TNF, or treated with IL-1. No significant inducible luciferase activity by TNF or IL-1 was detected with the shortest construct −93LUC used (Fig. 3, B and C). However, construct −174LUC, which in addition to the TATA-box, incorporates also the interferon regulatory factor site and proximal NF-κB site 3, mediated TNF and IL-1-inducible promoter activity as shown by a 3- to 4-fold increase in luciferase activity over the untreated control. Transfection of the −200LUC construct, containing an additional NF-κB site 2, resulted in a similar fold induction with the −174LUC construct, whereas the −247LUC construct, possessing all the potential NF-κB sites 1, 2, and 3, produced a significantly higher fold increase in luciferase activity than those observed with the −174LUC and −200LUC constructs following TNF or IL-1 treatment (11- to 12-fold induction in 293 and HeLa cells by TNF, 16-fold induction in HeLa cells by IL-1). When transfected with the four longer constructs, the level of luciferase activity did not show much variation, suggesting that the major elements mediating transcriptional activation of the c-IAP2 gene by TNF and IL-1 are located between positions −247 and −93, in which more than one NF-κB binding sites are functionally involved. The results were reproduced in many confirmatory experiments. With the −174LUC construct, stimulation of the luciferase activity in 293 cells by TNF over the untreated control ranged from the low of 2.5-fold to the high of 4.3-fold; in the experiments using the −247LUC construct, stimulation by TNF ranged from 8.4- to 14-fold.

Site-directed Mutagenesis Analysis of NF-κB Binding Sites Reveals That Two NF-κB Sites Are Required for TNF or IL-1-
mediated Transcriptional Activation of c-IAP2 Gene—Because our data indicated that TNF- or IL-1-inducible transcriptional activation of c-IAP2 gene expression could be directed by NF-κB binding elements residing in the 5′-flanking region between −247 and −93, we determined which of the potential NF-κB sites were functional. We introduced mutations into the −247LUC construct using the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s guidelines, generating four different constructs, each of which contained one or two mutated NF-κB sequences (Fig. 4A). The NF-κB mutant at site 2 retained the full TNF- or IL-1 inducibility as much as, or even higher, than that seen with the wild type −247LUC construct, whereas the double mutation at both NF-κB sites 1 and 3 resulted in loss of inducible promoter activity by TNF or IL-1 (Fig. 4B). These results indicate that both sites are functionally important for c-IAP2 gene expression could be directed by NF-κB binding elements residing in the 5′-flanking region between −247 and −93, thus indicating that the NF-κB site 1 is functional. Mutation of the NF-κB elements at sites 1 and 3 resulted in loss of inducible promoter activity by TNF or IL-1, whereas the double mutation at both NF-κB sites 1 and 3 resulted in loss of inducible promoter activity by TNF or IL-1.

Two Functional NF-κB Elements at Sites 1 and 3 Are Bound by the NF-κB p50/p65 Heterodimer Complex—To determine whether the nuclear proteins could bind to the NF-κB binding elements embedded in the c-IAP2 promoter, EMSA was performed with nuclear extracts of TNF-stimulated 293 or HeLa cells using specific oligonucleotides containing the NF-κB elements at sites 1, 2, and 3. Fig. 5A shows that 293 extracts treated with TNF for 30 min resulted in the induction of nuclear protein complex that bound to NF-κB elements at sites 1 and 3 but not to the site 2. Competition assays using excess unlabeled wild type oligonucleotides of sites 1 and 3 resulted in loss of the radioisotope-labeled bands, whereas the respective mutant oligonucleotides did not have any effect, confirming the binding specificity of nuclear proteins to the NF-κB sites 1 and 3.
results are consistent with those obtained from the mutational analysis of the respective NF-κB sites in which the NF-κB element at site 1 and site 3 were functional in driving the transcriptional activation following TNF stimulation. To characterize the nuclear protein complexes bound to the NF-κB sites 1 and 3, supershift assay was performed using antibodies against NF-κB proteins p50, p65, and c-Rel and an unrelated NF-IL6 antibody. Fig. 5B shows that preincubation with the p50 or p65 antibodies reduced the level of protein-DNA complexes of NF-κB sites 1 and 3. Furthermore, when pretreated with both antibodies, the protein-DNA complexes disappeared almost completely. However, antibodies recognizing c-Rel, another member of NF-κB proteins, and the unrelated NF-IL6 did not affect supershifting of proteins complexed with NF-κB sites 1 or 3. Essentially, similar results were obtained in experiments using HeLa cell nuclear extracts. Therefore, our results indicate that the NF-κB sites 1 and 3 could be bound by the NF-κB p50/p65 heterodimeric complex in both cell types.

**c-IAP2 Promoter**

**Is Activated by Overexpression of CD40 and Epstein-Barr Virus Oncoprotein LMP1**—Given that c-IAP2 mRNA is up-regulated by CD40 stimulation in B lymphocytes (19), we investigated whether overexpression of CD40 is capable of enhancing c-IAP2 promoter activity. We also investigated whether Epstein-Barr virus LMP1 can induce the c-IAP2 promoter activity. LMP1 is known to be involved in B cell transformation (25) and induces the antiapoptotic genes A20 and Bcl-2 through NF-κB activation (26, 27). 293 cells were transfected with the plasmids expressing CD40 or LMP1 along with either the −247LUC reporter construct or its mutated counterpart whose two functional NF-κB binding elements were destroyed (−247(mB1,3)LUC). In the case of transfection with the TNFR1 expression plasmid, 0.3 μg ofcrmA expression plasmid was added to protect cells from TNFR1-induced cell death. 24 h after transfection, cell lysates were prepared and processed for luciferase assays. The results shown are the average of three independent experiments.

**FIG. 6.** NF-κB-dependent activation of the c-IAP2 promoter by CD40 and LMP1. 293 cells were transfected with 1 μg of TNFR1, CD40, or LMP1 expression plasmid along with 0.5 μg of −247LUC reporter construct or with its mutated counterpart whose two functional NF-κB binding elements were destroyed (−247(mB1,3)LUC). In the presence of transfection with the TNFR1 expression plasmid, 0.3 μg of c-IAP2 promoter activation by CD40 and LMP1.

**DISCUSSION**

It is becoming evident that NF-κB appears to exert its antiapoptotic function through the induction of antiapoptotic genes, which include c-IAP1, c-IAP2, XIAP, Bcl2, IEX-1L, and A20 (4–10). Among members of the IAP family, c-IAP2 is particularly interesting in the sense that, first, not only is its expression under the control of NF-κB but also c-IAP2 conversely can activate NF-κB (10). Second, according to the previous reports, c-IAP2 is inducible by a variety of NF-κB-inducing stimuli, including TNF, IL-1, CD40, lipopolysaccharide, and etoposide in multiple cell lines, whereas induction of c-IAP1 and XIAP appears to be cell-type and stimulus-dependent (8–10, 18, 19, 29, 30). Despite such indication that c-IAP2 gene is preferentially up-regulated by NF-κB among the IAPs, direct evidence of transcriptional regulation of NF-κB on c-IAP2 is still missing. The purpose of this study was to clone and characterize the 5′-flanking region of the c-IAP2 gene and to identify the cis-regulatory elements responsible for transcriptional activation of the c-IAP2 gene.

Recently, the physical genomic map of c-IAP2 gene was reported by Young et al. (22). They determined the putative transcription start site by 5′-rapid amplification of cDNA ends
polymerase chain reaction and documented the partial sequence information upstream of their proposed start site. In our studies, however, the 2.1-kb segment upstream of the proposed transcription start site did not exhibit basal promoter activity as well as NF-κB-dependent reporter gene activation, instead, the 1.4-kb segment downstream from the proposed transcription start site could direct the transcriptional activation of the c-IAP2 gene following stimulation of cells with TNF or transfection of NIK or p65 (Fig. 1). In addition, the downstream region from the proposed start site featured a number of elements typical to the eukaryotic promoter including a stream region from the proposed start site estimated to be longer than 3.5 kb. Several potential cis-acting enhancer elements have been identified in the sequence of the 5′-flanking region of the c-IAP2 gene. The transfection experiments of the promoter deletion constructs linked to the luciferase gene revealed that the region conferring TNF- or IL-1-inducible promoter activation resides between −247 and −93 of the c-IAP2 promoter. Site-directed mutational analysis and EMSA of the putative NF-κB binding sites incorporated within the −247 region of the promoter identified that two NF-κB binding elements at positions −147 and −210 are required for promoter activity and that they function cooperatively in mediating transcriptional activation of the c-IAP2 gene. In addition, supershifting experiments with antibodies against NF-κB subunits characterized that the two NF-κB binding sites could be recognized and bound by the NF-κB p50/p65 heterodimeric complex. Therefore, our results provide direct mechanistic evidence of transcriptional regulation of NF-κB on c-IAP2 gene and a confirmatory proof of the notion that NF-κB functions as a primary factor in the regulation of genes involved in cell survival or protection in response to apoptotic stimuli. Despite the predominant role of NF-κB in c-IAP2 gene expression as characterized by the present study, a recent report suggests that induction of c-IAP2 gene is not solely dependent on NF-κB activation (34). c-IAP2 can be induced by dexamethasone and, to a higher extent, by treatment of dexamethasone plus interferon-γ in a lung carcinoma cell line, A549, in the setting that dexamethasone protects the cells from apoptosis induced by interferon-γ and anti-Fas antibody (34). These results suggest that dexamethasone and interferon-γ act in synergy to induce c-IAP2. In this regard, it is of interest that a glucocorticoid response element at position −514 as well as interferon regulatory factor-1 inducible interferon-γ binding sites at position −130 and −475 were identified by computer search in the 5′-flanking promoter region of the c-IAP2 gene. Whether those elements can function as specific binding sites in the context of the c-IAP2 5′-flanking DNA remains to be elucidated. In addition, according to a recent report (35), stimulation of T cell receptor (TCR) can also induce c-IAP2 in the T cell line D11/27. It showed that the TCR-mediated induction of c-IAP2 gene was slower but stronger than that mediated by TNF. In their study, endogenously produced TNF and lympho-toxin α in the culture medium appeared not to be involved in the TCR-mediated c-IAP2 induction, because the respective neutralizing antibodies did not affect the level of c-IAP2 mRNA. It is well established that TCR-mediated transcriptional regulation that initiates cytokine gene expression requires several signaling pathways, which activate different transcription factors simultaneously. For example, TCR-induced activation of AP-1, NF-κB, and NF-κB all cooperate to enhance IL-2 gene transcription (36). Therefore, it is noteworthy that NFAT-dependent elements are incorporated in the 5′-flanking sequence of c-IAP2. Whether NFAT plays a role in the c-IAP2 promoter during TCR stimulation remains to be elucidated. In this sense, the present characterization of the 5′-flanking sequence of the c-IAP2 gene makes it possible to direct future efforts at a precise identification of the cis-acting elements and corresponding transcription activating factors involved in the TCR- or dexamethasone-mediated c-IAP2 gene induction.

Another noteworthy finding in this study is that overexpression of CD40 or LMP1 resulted in the c-IAP2 promoter-driven reporter gene activation. CD40 and LMP1 are cell surface receptors triggering NF-κB activation and, consequently, up-regulating the antiapoptotic proteins Bcl-2 and A20 (26, 27, 37). Along with the report that CD40 stimulation on B cells differentially increased the steady-state level of c-IAP2 mRNA, although not significantly inducing the levels of other family members (XIAP, NAIP, and c-IAP-1) (19), our demonstration that CD40 activated the c-IAP2 promoter suggests that one mechanism by which CD40 rescues cells from apoptosis is via up-regulation of c-IAP2. Also our results suggest that LMP1, an Epstein-Barr virus (EBV) latent membrane protein responsible partly for B cell transformation, mediates enhanced expression of c-IAP2 mRNA. In these regards, it is very interesting that LMP1 proteins are present in the Reed-Sternberg cells in more than 70% of Hodgkin’s disease cases (38) and that the high level of c-IAP2 expression is seen in the Reed-Sternberg cells (39). Also of interest is that TRAF1 is shown to be overexpressed in the Reed-Sternberg cells and EBV-transformed lymphoid cells (40). Furthermore, TRAF1 can be induced by LMP1 in the B lymphocyte cell line (41), and its overexpression inhibits antigen-induced CD8+ T lymphocyte apoptosis (42). As indicated, TRAF1 is associated with the protection of apoptosis induced by TNF under the circumstances in which TRAF1, TRAF2, c-IAP-1, and c-IAP2 are all expressed (42). Therefore, it can be speculated that the LMP1-inducible c-IAP2 protein functions as a prosurvival or antiapoptotic factor in certain EBV-associated lymphoid cell proliferation or transformation such as those seen with the Reed-Sternberg cell, acting independently or in concert with TRAF1 or other LMP1-inducible antiapoptotic proteins such as A20 and Bcl-2. Further investigations are necessary to clarify the role of c-IAP2 in EBV-associated lymphoproliferation and in Hodgkin’s disease.

Taken together, the data presented here confirm the antiapoptotic protein c-IAP2 as a target of NF-κB signaling, the expression of which is tightly dependent on the presence of two NF-κB binding elements within the promoter. We found c-IAP2 to be induced by stimulation of a variety of cell surface receptors, including TNFR1, IL-1 receptor, CD40, and LMP1, which trigger NF-κB activation. Therefore, the present elucidation of the transcriptional regulating mechanism of the c-IAP2 gene expression provides an additional proof of the notion that transcriptional activation of NF-κB on antiapoptotic genes is a common mechanism involved in the survival or protective effect in response to various apoptotic stimuli. This also sheds more light on the role of c-IAP2 in tumor cell survival, transformation, and anticancer drug resistance.
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J. Biol. Chem. 2000, 275:18022-18028.
doi: 10.1074/jbc.M001202200 originally published online April 6, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001202200

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