Cell Death and Differentiation (1999) 6, 314–325

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# A novel adenovirus E1B19K-binding protein B5 inhibits apoptosis induced by Nip3 by forming a heterodimer through the C-terminal hydrophobic region

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Received 16.7.98; revised 13.11.98; accepted 17.12.98
Edited by Y. Kuchino

## Abstract

**The adenovirus E1B19K protein inhibits apoptosis induced by E1A and other divergent signals. The cellular proteins that interact with E1B19K have been analyzed by isolating cDNA clones by the yeast two hybrid system. One of these clones encodes B5 which consists of 219 amino acid residues and contains the putative BH3 and transmembrane regions. B5 binds strongly to Nip3 and itself, weakly to E1B19K, but not to Bcl-2 and localizes in nuclear envelope, endoplasmic reticulum and mitochondria. B5 has sequence homology with Nip3 in the middle and C-terminal regions, but not in the N-terminal region. Unlike other E1B19K binding BH3 proteins so far characterized, B5 does not induce apoptosis, but inhibits apoptosis induced by Nip3. However the deletion mutant B5Δ1-31 lacking the N-terminus does induce apoptosis, although weaker than does Nip3, suggesting that the N-terminal region is masking the apoptosis-inducing capacity of B5.**

**Keywords:** apoptosis; E1A; E1B19K binding protein B5; Nip3

**Abbreviations:** AT, aminotriazole; BH, Bcl-2 homology; FCS, fetal calf serum; NP40, Nonidet P40; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride

## Introduction

The adenovirus E1A gene product E1A has abilities to induce cell proliferation and death and to inhibit cell differentiation depending on cellular factors to which it interacts. The E1B19K protein (E1B19K) encoded by the adjacent E1B gene suppresses E1A-induced apoptosis and facilitates viral growth in the infected cells[1,2] and transformation of primary rodent cells by E1A.[3,4] E1B19K shares limited sequence homology with Bcl-2 and with other Bcl-2 family members within Bcl-2 homology region (BH) 1 and just before BH2[5,6] and inhibits apoptosis induced by tumor necrosis factor $\alpha$ and Fas ligand even more efficiently than does Bcl-2.[3,6–8]

E1B19K has been shown to interact with and disrupt the organization of intermediate filaments and modify the structure of nuclear laminA and laminC.[9] The cDNA clones encoding the E1B19K-binding proteins Nip1, Nip2, Nip3,[5] Bax, Bak[10–14] and Nbk/Bik,[15,16] have been isolated by the yeast two hybrid system. Bax and Bak are the members of the Bcl-2 family, but, unlike Bcl-2 and Bcl-XL, they are inducers of apoptosis. Most of the Bcl-2 family members share BH1, BH2 and BH3 and the C-terminal transmembrane region which directs their localization to mitochondrial outer membrane, nuclear envelope and endoplasmic reticulum.[17–19] Among Nip1, Nip2 and Nip3, Nip3 has been shown to be a dimeric mitochondrial protein and to induce apoptosis in Rat-1 fibroblasts and MCF-7 breast carcinoma.[20] Nbk/Bik is also a potent stimulator of apoptosis.[16] Both Nip3 and Nbk/Bik lack BH1 and BH2, but contain putative BH3. A Nip3 mutant lacking the C-terminal transmembrane domain loses the ability to dimerize, localize to mitochondria and induce apoptosis.[20]

We have previously established a cell line MA1 from human epidermoid carcinoma cells KB by introducing the adenovirus type 2 E1A 12S cDNA linked to the mouse mammary tumor virus long terminal repeat (MMTV-LTR). MA1 cells express E1A12S in response to dexamethasone (dex) and undergo apoptosis after stabilization of wild type (wt) p53 and degradation of topoisomerase II$\alpha$ through modification of the ubiquitin-proteasome pathway.[21,22] Both induction of apoptosis and degradation of topoisomerase II$\alpha$ were inhibited by the E1B19K and Bcl-2 in a dose-dependent manner.

To analyze the role of E1B19K in the suppression of apoptosis in MA1 cells, cDNAs encoding the E1B19K-binding proteins have been isolated by using the yeast two hybrid system. The B5 protein encoded by one of these clones contains putative BH3 and transmembrane domains in the middle and C-terminal regions, and localizes to nuclear envelopes, endoplasmic reticulum and mitochondria. B5 has sequence homology with Nip3 in these regions, but has unique sequence in the N-terminal region. B5 itself has no ability to induce apoptosis, but rather inhibits apoptosis induced by Nip3 in KB cells. However, B5 is capable of inducing apoptosis when it lacks the N-terminal region, although the extent of apoptosis induced is smaller than that induced by Nip3. These results suggest that B5 is a unique negative regulator of

apoptosis among the E1B19K binding BH3 proteins and its capacity to induce apoptosis is masked by the N-terminal region.

## Results

### Isolation of cDNA clones encoding E1B19K-binding proteins by the yeast two hybrid system

To analyze the biological function of E1B19K to inhibit apoptosis induced by E1A in MA1 cells in a dose-dependent manner, the cDNA clones encoding E1B19K binding proteins were isolated by the yeast two hybrid system. Tester strains were established from yeast strains HF7C and Y190 by introducing pGBT-B19 which express a fusion protein composed of Gal4 DNA binding domain and E1B19K. A cDNA library was constructed from MA1 cells in the pGAD424 vector, which directs the synthesis of fusion proteins composed of cDNA-encoded polypeptides and the Gal4 transcriptional activation domain. The tester strains HF7C-19K and Y190-19K were transformed with the pGAD-MA1 cDNA library and plated on medium lacking histidine. $His^+$ colonies developed (about 250 clones) were then tested for production of $\beta$-galactosidase by an X-Gal filter assay. Thirty-two clones were finally selected and cDNA sequences of these clones were partially determined by using the primer which anneal within the 3′-end portion of the Gal4 activation domain. The cDNAs were classified into five groups and one to two representative cDNAs in each group were recloned into pBluescript KS(+) and sequenced. Twenty-six clones were either laminA or laminC, two each clones contained Bak and novel sequences and one each clone was Nip2 and Nip3. The two clones containing novel sequences were turned out to be the same and the protein encoded was termed B5.

B5 cDNA comprises 1526 base pairs (bp) and contains a continuous open reading frame of 219 amino acids (aa) (Figure 1A). No B5 cDNA clones that contain longer coding sequence could be isolated from a MA1 cell cDNA library constructed with the $\lambda$ZapII expression vector. The first ATG codon has the Kozak motif and the protein encoded was efficiently expressed, when the cDNA was cloned in an expression vector as shown in later sections. B5 has high aa sequence homology with the E1B19K-binding protein Nip3 in the middle and C-terminal regions as shown by underlines in Figure 1A, but has the unique sequence in the N-terminus from positions 1–34. The amino acid sequences of B5 and Nip3 within these homologous regions are compared in Figure 1B. More than 80% of the B5 amino acid residues from positions 60–96 are identical or functionally-related with the corresponding residues of Nip3. B5 has no BH1 and BH2 domains, but has the BH3-like domain from positions 130–144. The amino acid residues of B5 within this domain differ considerably from those of Nip3 and the Bcl-2 family members, but the critical amino acids, leucine[134], aspartic acid[139] and valine[141] instead of isoleucine are conserved as compared with the BH3 domain of Bak.[23] The hydropathy plots predicted that the aa sequence from positions 176–204 is a membrane-spanning region which is characteristic of the Bcl-2 family members. The amino acid residues of B5 within this putative transmembrane region are identical or functionally-related with those of Nip3 except one residue. A search of the protein data base (GenBank, accession number 2511529) with the B5 sequence revealed that B5 was listed in a group of genes expressed in an infant brain.

### Interaction of B5 with other E1B19K-binding proteins in yeasts

To see the interaction of B5 with other E1B19K-binding proteins, pGAD424 expression vectors encoding the Gal4 transactivation domain fused to laminA, laminC, Nip2, Nip3, Bak, Bcl-2 or B5 were constructed. Each of these vectors were introduced into HF7C yeast cells with the pGBT9 expression vector encoding the Gal4 DNA binding domain fused to B5 and the transformants were streaked on S.D. medium lacking histidine, but containing 3 mM 3-AT. Under the conditions, the growth was dependent on the interaction of B5 with the other E1B19K-binding proteins. The result is summarized in Figure 2A. B5 showed strong interaction with Nip2, Nip3 and B5 itself, but no interaction with Bak and Bcl-2. Nip2 and Nip3 interacted strongly with Bcl-2 as previously reported.[5] B5 interacted weakly with E1B19K and laminC, but not with laminA, although E1B19K interacts strongly with laminA and laminC.

The B5 mutant lacking the C terminus ($\Delta$170–219) could not interact with E1B19K, Nip3 and B5 itself (Figure 2B), suggesting that the interaction requires the putative transmembrane region. The B5 mutant lacking codons 58–96 interacted with Nip3 and B5 itself but not with E1B19K, indicating that the middle region having high sequence homology to Nip3 is also required for interaction with E1B19K. The interaction of E1B19K and B5 was similarly analyzed with E1B19K mutants containing a deletion either in the N-terminus ($\Delta$1–22) or in the middle region ($\Delta$90–96) that are essential for suppression of the E1A-induced apoptosis. These mutants were unable to interact with B5, however, a point mutation at the aa residue 82 (DY), which also abolish the E1B19K suppressive activity[24] had no effect on the interaction of E1B19K with B5. Interaction of B5 with Nip3 and E1B19K was also analyzed by $\beta$-galactosidase assay (Figure 2D). The $\beta$-galactosidase activities in HF7C cells transformed by pGBT-B5 and pGAD-B5 or pGAD-Nip3 were higher than those in the cells transformed by pGBT-E1B19K and pGAD-B5 or pGAD-Nip3 indicating that the interaction of B5 with Nip3 and B5 itself is stronger than that of E1B19K with Nip3 and B5. The result obtained is consistent with that obtained by the histidine selection.

### Homo- and hetero-dimerization of B5 *in vivo*

To see homo- and hetero-dimerization of B5 *in vivo* and to determine the domain required for dimerization, the expression vector pCXN-FB5 encoding Flag epitope-tagged B5 (FB5) was used to transfect COS7 cells, since the vector contains the SV40 ori and multiplicates in COS7 cells expressing SV40 large T antigen. The vectors, pCXN-

FB5ΔC encoding B5 lacking the C-terminus (Δ188–219) and pCXN-FB5ΔNM lacking the N-terminal and middle regions (Δ1–119) were similarly transfected (Figure 3A). The lysates prepared at 20 h after transfection were analyzed by Western blotting with anti-Flag antibody. Two proteins of 35 and 70 kDa that correspond to monomeric and dimeric form of B5

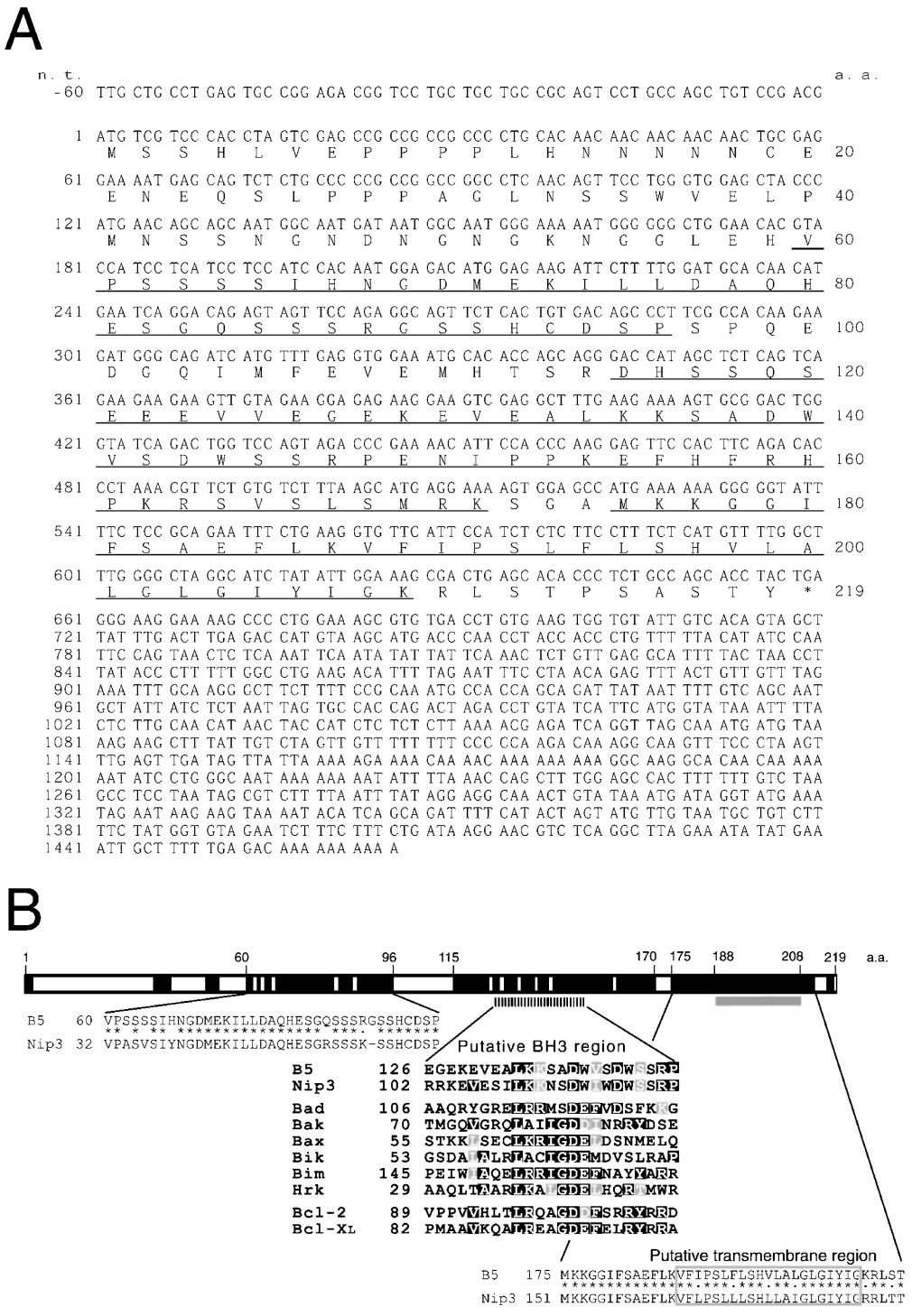


**Figure 1** Structural features of B5. (**A**) Nucleotide sequence and the predicted amino acid (aa) sequence of B5. The aa sequences highly homologous with those of Nip3 are underlined. (**B**) Comparison of amino acid sequences between B5 and Nip3 within the highly homologus regions. The B5 sequences from positions 60–96, from positions 115–170 containing putative BH3 domain and from positions 175–213 containing putative transmembrane region are compared with the corresponding sequences of Nip3. The amino acid residues that are identical are shown by stars and those that are functionally-related are shown by dots. The B5 putative BH3 sequence is also compared with the BH3 core sequences of the Bcl-2 family members.[28] The identical residues are black-boxed and functionally related residues are dark-boxed

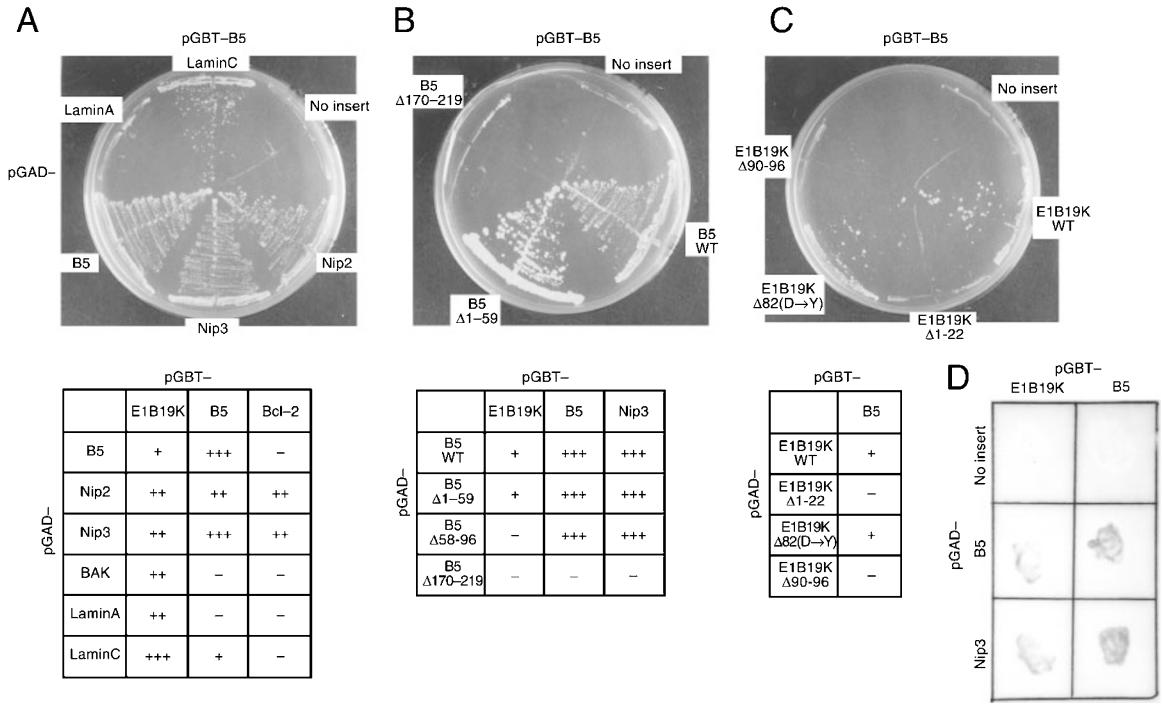


**Figure 2** Interaction of B5 with E1B19K binding proteins in yeast strain HF7C. (**A**) The pGBT9 expression vector encoding the Gal4 DNA binding domain fused to B5 was transformed with the pGAD424 expression vectors encoding the Gal4 transactivation domain fused to either Nip2, Nip3, B5, Bak, Bcl-2, laminA or laminC as indicated. The transformants grown on plates containing histidine but lacking leucine and tryptophan were streaked on plates not containing histidine but containing 3 mM 3AT. A part of the result showing the growth of colonies is shown above. Interaction of E1B19K and Bcl-2 with the E1B19K binding proteins was similarly analyzed as controls and the results obtained are summarized in the Table shown below. The affinity of binding shown is as follows; +++, very strong affinity; ++, strong affinity; +, weak affinity; −, no affinity. (**B**) Interaction of B5 mutants with E1B19K binding proteins. The pGBT9 expression vectors for B5 deletion mutants fused to the Gal4 DNA binding domain were introduced with the pGAD424 expression vectors encoding the fusion proteins of E1B19K, B5 or Nip3 into yeast strain HF7C, and the interaction was analyzed similarly as described in **A**. (**C**) Interaction of B5 with E1B19K deletion mutations. The interaction was similarly analyzed. (**D**) pGBT-B5 and pGBT-E1B19K were cointroduced with pGAD-B5 and pGAD-Nip3 into HFC7 cells as indicated. Colonies developed were streaked on the filter placed on S.D., $His^-$, 3AT agar medium and assayed for $\beta$-galactosidase after incubation for 2 days

were detected in FB5 lysate. In FB5ΔNM lysate, two proteins of 15 and 30 kDa were detected. These sizes just correspond to the monomeric and dimeric form of the C-terminal half of B5. Only one protein of 32 kDa, which corresponds to B5 lacking the C-terminus, was detected in FB5ΔC lysate. The result suggests that B5 dimerizes *in vivo* through the C-terminal transmembrane region. Essentially the same result was obtained with 293T cells expressing SV40 large T antigen (data not shown).

Hetero-dimerization of B5 with Nip3 *in vivo* was similarly analyzed by immunoprecipitation-Western blot analysis (Figure 3B, top). COS7 cells were transfected with pCXN-FB5, pCXN-HA-Nip3 or both. pCXN-HA-Nip3 encodes HA epitope-tagged Nip3. The lysates prepared at 24 h after transfection were first blotted with anti-HA antibody which detects the presence of Nip3. In Nip3 lysate, Nip3 was detected as two bands of 30 and 60 kDa. Although a predicted molecular mass of Nip3 is 21.5 kDa (194 aa), these bands are likely to correspond to a Nip3 monomer and a dimer as also observed by Chen *et al*.[19] An additional band of 65 kDa was detected in the B/N lysate prepared from the cells transfected with B5 and Nip3 expression vectors. The band seems to correspond to the B5/Nip3 heterodimer, because the molecular mass of B5 (219 aa) is slightly larger than that of Nip3. No Nip3 was detected in the B5 lysate. Analysis of the immunoprecipitates prepared from the B/N lysate with anti-Flag antibody by Western blotting with anti-HA antibody revealed three specific bands of 30, 60 and 65 kDa. None of these bands were detected in the immunoprecipitates prepared from the B5 and Nip3 lysates. The 65 and 60 kDa proteins correspond to the B5/Nip3 heterodimer and the Nip3/Nip3 homodimer. The Nip3/Nip3 homodimer might be coprecipitated with the B5/B5 homodimer and/or the B5/Nip3 heterodimer. The 30 kDa protein corresponds to the Nip3 monomer which might be generated by dissociation of the B5/Nip3 heterodimer and/or the Nip3/Nip3 homodimer during immunoprecipitation and Western blotting procedures. To compare the size of putative B5/Nip3 heterodimer with that of B5 homodimer, the immunoprecipitates prepared with anti-Flag antibody were blotted with the same anti-Flag antibody (Figure 3B, bottom). Two proteins of 35 and 70 kDa were detected in the immunoprecipitates prepared from both B5 and B/N lysates but not from the Nip3 lysate. The 35 kDa protein corresponds to the B5 monomer and

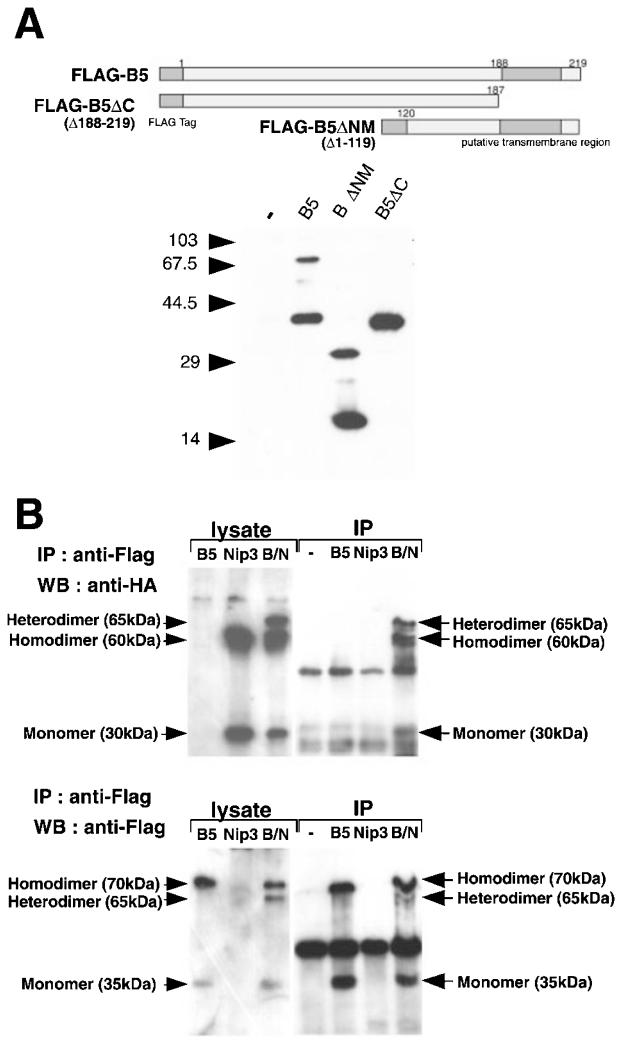


**Figure 3** Homo- and hetero-dimerization of B5 *in vivo*. (**A**) Homodimerization of B5. COS7 cells were transfected with the expression vectors, pCXN-FB5, pCXN-FB5ΔNM or pCXN-FB5ΔC, each encoding Flag epitope-tagged B5 or its deletion mutant as shown above. The lysates prepared at 20 h after transfection were analyzed by Western blotting with anti-Flag mouse monoclonal antibody M5. (**B**) Heterodimerization of B5 with Nip3. COS7 cells were transfected with pCXN-FB5, pCXN-HA-Nip3 or both and the lysates prepared at 24 h after transfection were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were analyzed by Western blotting with either anti-HA antibody (top) or with anti-Flag antibody (bottom)

the 70 kDa protein to the B5 homodimer. In addition to these bands, the band of 65 kDa, presumably the B5/Nip3 heterodimer, was detected in the B/N immunoprecipitate, although its amount was much less than that of the 70 kDa B5/B5 homodimer. The reason is presently unclear, since B5 binds to both Nip3 and B5 itself strongly. These three bands were also detected in the B/N lysate by Western blotting. The homo- and hetero-dimerization of B5 is likely to occur through putative transmembrane region. It has been recently shown that LXXLL-containing motifs are required for hydrophobic interaction of two proteins.[25] Nip3 contains this motif and B5 contains LXXVL sequence (Figure 1B).

## Subcellular localization of B5

To see the subcellular localization of B5 and to analyze the B5 domain required for interaction with E1B19K, both KB and COS7 cells on cover slips were transfected with either pCXN-FB5, pCXN-FB5ΔC or pCXN-FB5ΔNM (Figure 3A) together with the E1B19K expression vector. The cells were fixed at 24 h after transfection and the localization of B5 and E1B19K was analyzed by indirect immunofluorescence. Anti-Flag mouse monoclonal antibody and anti-E1B19K rabbit polyclonal antibody were used as primary antibodies and visualized with fluorescein and Texas Red-conjugated secondary antibodies, respectively (Figure 4). B5 was expressed in the nuclear envelope/endoplasmic reticulum (ER) region, and in cytoplasmic patches, while E1B19K localized primarily in the nuclear envelope/ER region in both KB (Figure 4A) and COS7 cells (Figure 4B). This different localization of B5 and E1B19K became apparent, when both images were merged to yield yellow color. The yellow color was restricted to the nuclear envelope/ER region, while green color remained in cytoplasmic patches. These patches are typical of a mitochondrial distribution. In contrast, B5ΔC lacking the C-terminal putative transmembrane region localized in the cytoplasm uniformly especially in COS7 cells. Although the intense green color was observed in the nuclear envelope of KB cells, very little yellow color was visible in both KB and COS7 cells, indicating that B5ΔC lost the ability to colocalize with E1B19K. The localization of B5ΔNM lacking the N-terminal and middle regions was essentially the same as that of B5.

To confirm the mitochondrial localization of B5, COS7 cells were transfected with pCXN-FB5 and treated with FITC-conjugated MitoTracker which binds to the mitochondrial inner membrane 30 min prior to fixation. The cells were then stained with anti-Flag mouse monoclonal antibody and Cy3-conjugated anti-mouse IgG. The localization of B5 to mitochondria was evident, since the pattern of Cy3 localization (Figure 5A) was identical with that of MitoTracker (Figure 5B). The overlapping of these images yielded the same pattern of yellow color (Figure 5C).

## Expression level of Nip3 increased steeply, while that of B5 unchanged during induction of apoptosis in MA1 cells

To see the changes in expression levels of the gene encoding E1B19K binding proteins during induction of apoptosis in MA1 cells, poly (A)-containing RNAs were prepared from MA1 cells after treatment with dex for various times and aliquots of them were analyzed by Northern blotting (Figure 6). Time course of the apoptotic process in MA1 cells is shown on the top of the Figure. In brief, after induction of E1A expression, p53 stabilizes within 24 h and topoisomerase IIα begin to be degraded via the ubiquitin-proteasome pathway. The loss of cell viability begin to be observed subsequently.[21,22] Two species of B5 mRNA with the lengths of 4.0 and 1.5 kilobases (kb) were detected (Figure 6Ba). The levels of these mRNAs, however, remained unchanged during the apoptotic process. The level of Bak mRNA began to increase after 24 h and increased 2–3-fold at 48 h (Figure 6Bb). The level of Nip3

mRNA increased steeply along with the progression of the apoptotic process, reaching a maximal level of more than tenfold higher than the original at 48 h (Figure 6Bd). The level of laminA mRNA was unchanged, while that of laminC mRNA increased after 36 h (Figure 6Be). Under the conditions, the level of $\beta$-actin mRNA was unchanged (Figure 6Bc and f).

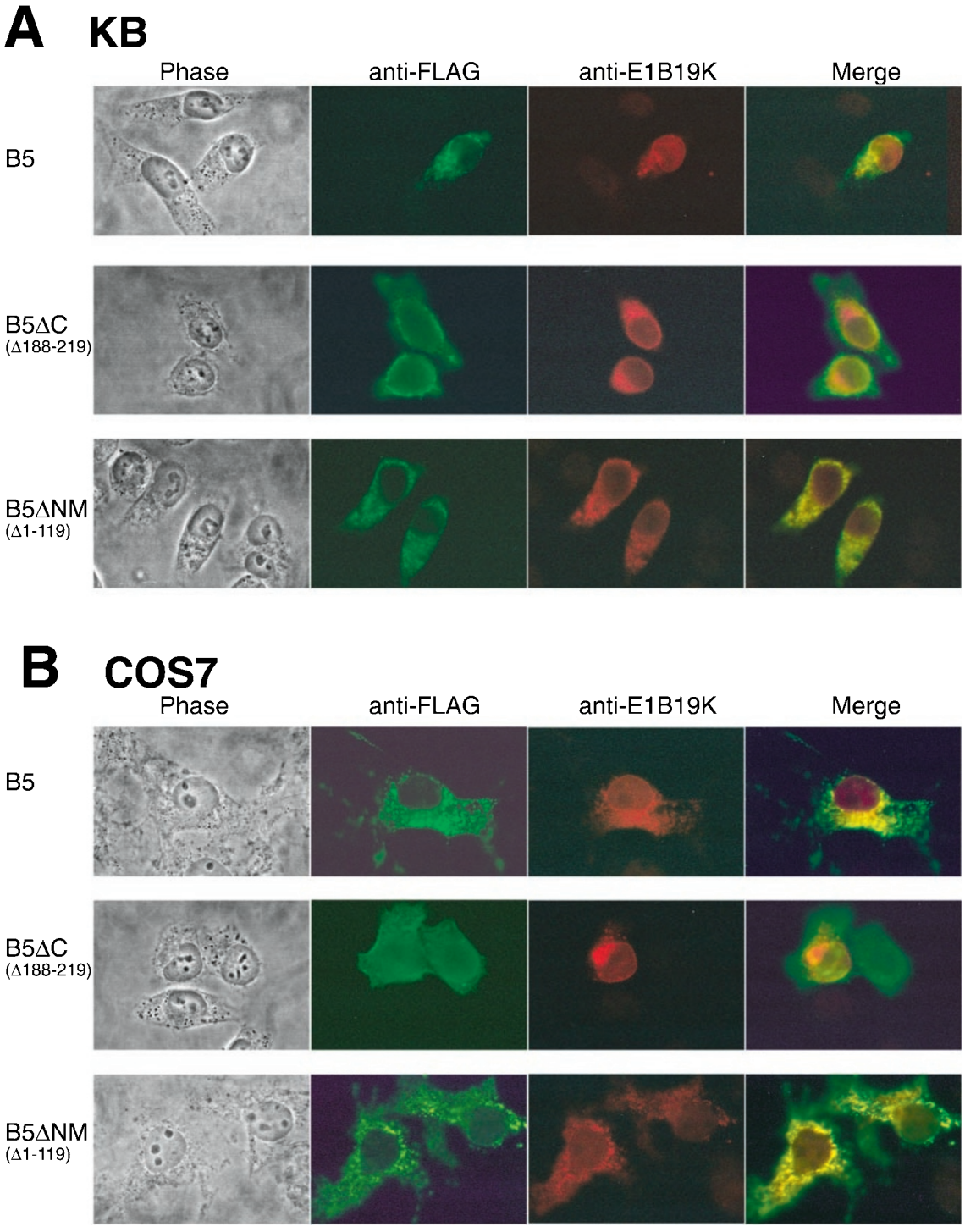


**Figure 4** Intracellular localization of B5. KB (**A**) and COS7 cells (**B**) on coverslips were transfected with pCXN-FB5, pCXN-FB5ΔC, or pCXN-FB5ΔNM together with pH$\beta$APr-E1B19K and fixed at 16 h after transfection. The cells were permeabilized with 0.1% Triton X and incubated with anti-Flag antibody M5 or with anti-E1B19K rabbit polyclonal antibody raised against the C-terminal 16 amino acid residues at 1 : 250 dilutions. The cells were then incubated with FITC-conjugated anti-mouse IgG goat antibody (CAPPEL) or Texas Red-conjugated anti-rabbit IgG donkey antibody (Amersham) at 1 : 1000 dilutions. Photographs were taken at a magnification of $\times$1000

### Inhibitory effect of B5 on the induction of apoptosis by Nip3 is caused through the C-terminal region

To analyze the ability of B5 to induce apoptosis, KB cells were transfected with the expression vectors for E1A, Nip3, B5 and combinations of these vectors, together with expression vector for CD20, a cell surface calcium binding protein. The apoptotic cells were quantitatively assayed using FACS by the accumulation of cells with DNA of a sub-G1 content earlier after transfection (20 h after transfection) to minimize the development of cell damage caused by transfection (Figure 7). The cell damage caused by transfection procedure in control cells (−), to which only the CD20 expression vector was transfected (Figure 7Aa), resulted in the increase in the sub-G1 population to 4.1%. The sub-G1 population did not increase significantly by transfection with the B5 expression vector but increased steeply by transfection with the Nip3 expression vector (Figure 7Ab and c). Transfection with the E1A expression vector resulted in the increase in the sub-G1 population to 10.9% and this increase was almost unaffected by cotransfection with the B5 expression vector. Cotransfection with expression vectors for E1A and Nip3 (Figure 7Af) showed the sub-G1 increase to 29.8%, but no additive increase was observed, indicating that the action of Nip3

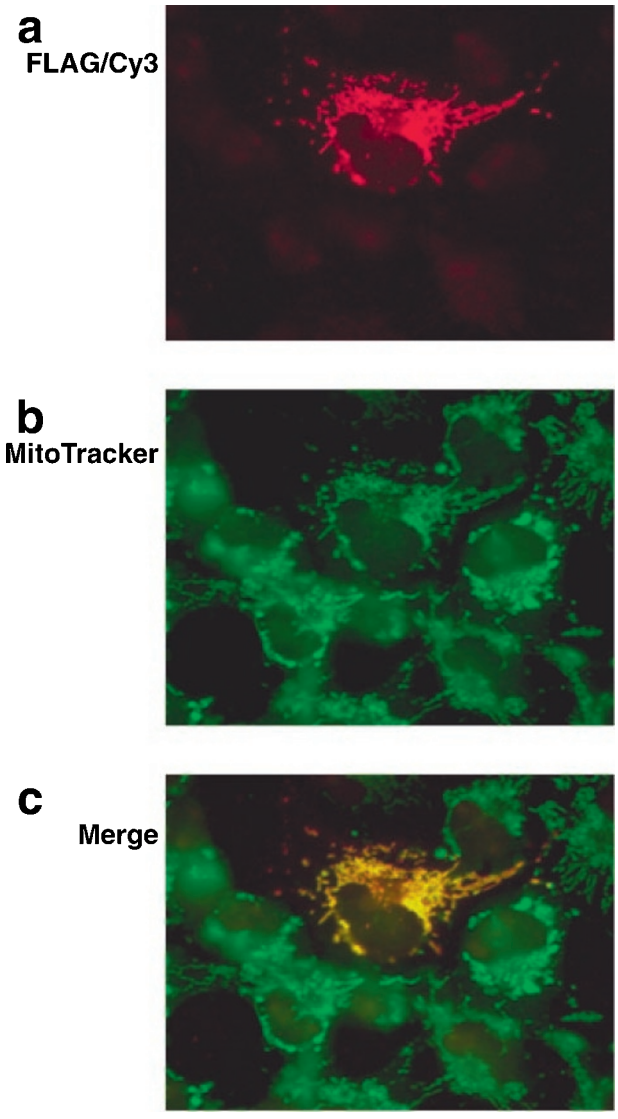


**Figure 5** Localization of B5 to mitochondria. COS7 cells were transfected with pCXN-FB5 and stained with FITC-conjugated MitoTracker which binds to the mitochondrial inner membrane (**B**). The cells were then stained with anti-Flag mouse antibody M5 and Cy3-conjugated anti-mouse IgG antibody (**A**). The above images were combined (**C**). A uniform yellow color generated indicates the localization of B5 to mitochondria

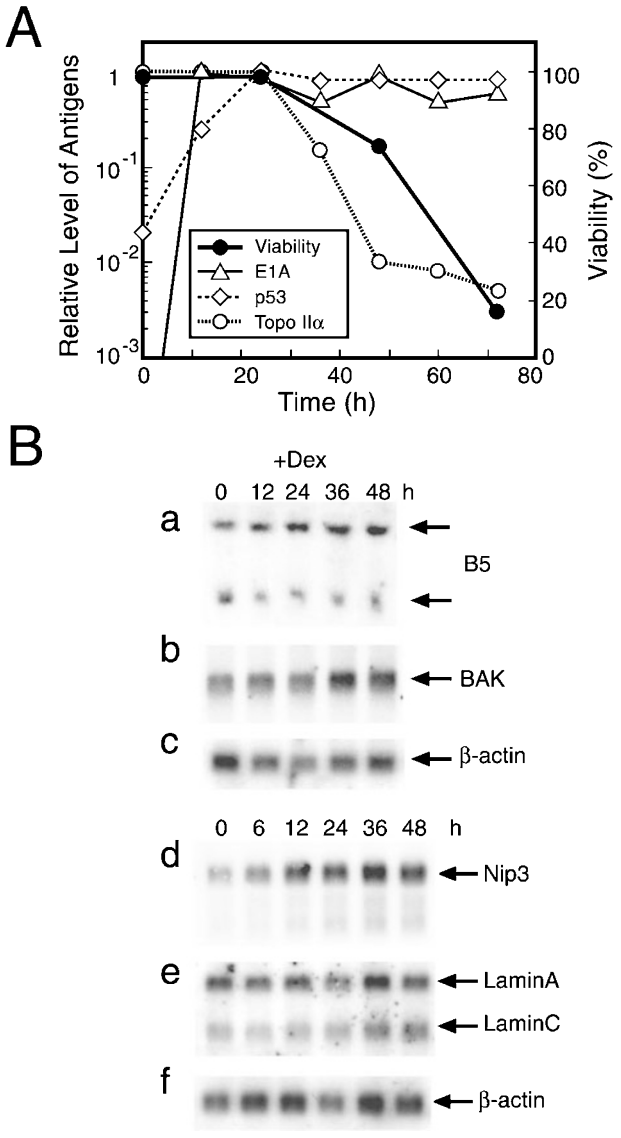


**Figure 6** (**A**) Time course of the induction of apoptosis in MA1 cells after treatment with dex is shown together with those of p53 stabilization and topoisomerase II$\alpha$ degradation. (**B**) Poly (A)-containing RNAs were prepared from MA1 cells treated with 1 $\mu$M dex at the times indicated by using oligotex(dT)30.[36] Aliquots of 3 $\mu$g of RNAs were electrophoresed and the expression levels of B5 (**a**), Bak (**b**), Nip3 (**d**) and lamin A and C (**e**) mRNAs were analyzed by Northern blotting with $^{32}$P-labeled cDNAs as indicated on the right

predominates over that of E1A, presumably because the induction of apoptosis by E1A takes longer than does Nip3. When the cells were cotransfected with expression vectors for E1A and E1B19K, the increase in the sub-G1 population

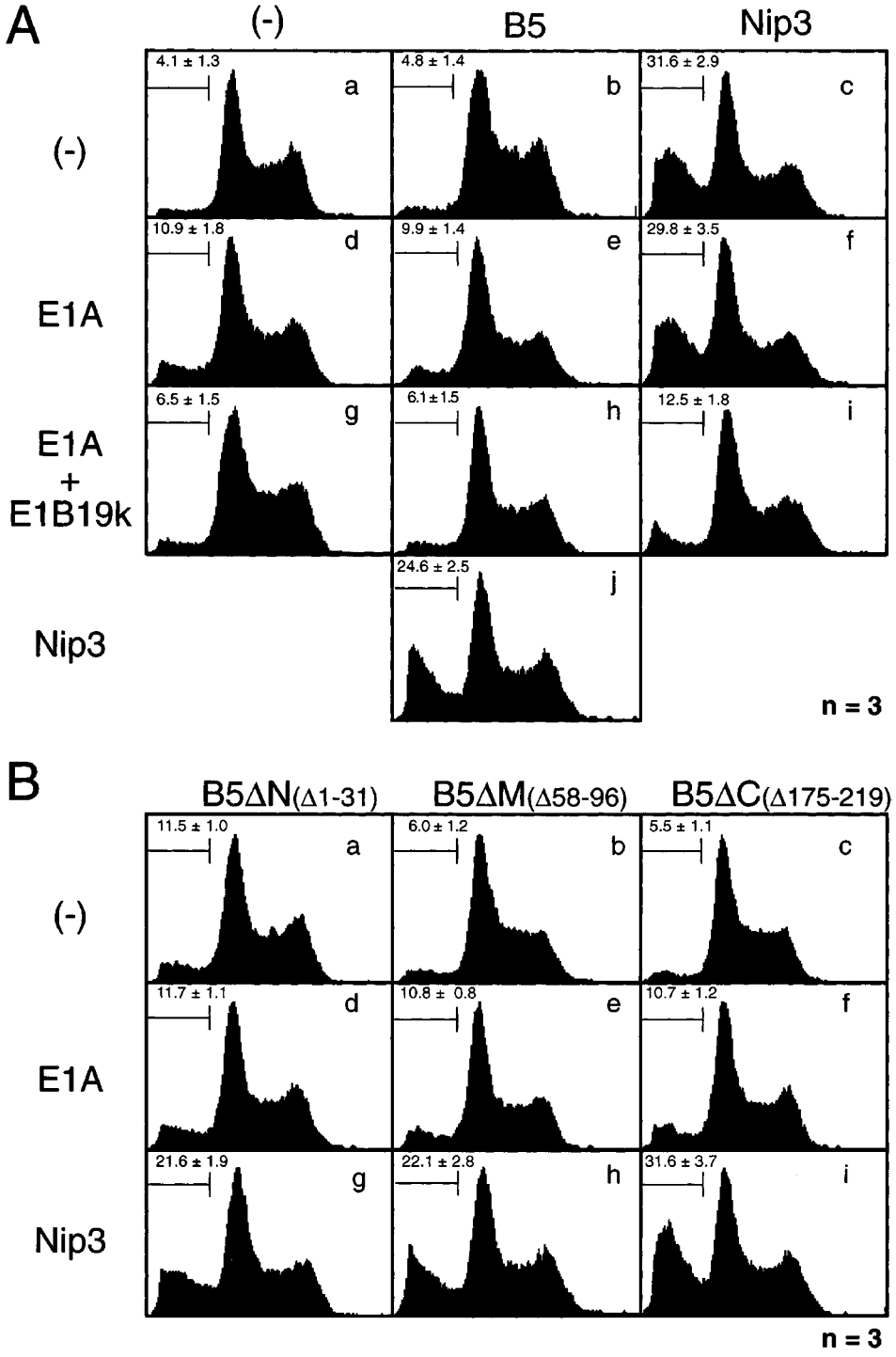


**Figure 7** Induction of apoptosis by Nip3 is suppressed by coexpression with B5. (**A**) Growing cultures of KB cells in 3.5 cm-dishes were transfected with 0.3 $\mu$g each of the expression vectors, pH$\beta$APr-B5, pH$\beta$Apr-Nip3, pH$\beta$APr-E1A12S, pH$\beta$APr-E1B19K and combinations of these vectors as indicated together with 0.1 $\mu$g of pCMV-CD20. A total amount of the vectors was adjusted to 1.0 $\mu$g per dish with pH$\beta$APr-1. At 20 h after transfection, the cells were stained with PI and DNA contents of the cells were analyzed by flowcytometry. The cells were gated based on the expression of CD20. The sub-G1 population was shown as the percentage of the total cell population. The mean values of the three experiments ($n$=3) are shown with standard deviation. (**B**) Effect of B5 deletion mutants on E1A- and Nip3-induced apoptosis. KB cells were transfected with pH$\beta$Apr-E1A12S or pH$\beta$Apr-Nip3 together with either pH$\beta$APr-B5$\Delta$N, pH$\beta$APr-B5$\Delta$M or pH$\beta$APr-B5$\Delta$C as indicated. DNA contents of the cells were analyzed as described in (**A**)

caused by E1A was partially inhibited from 10.9 to 6.5% (Figure 7Ag). This decrease corresponds to about 65% inhibition, when calculated after subtraction of the background value, and was unaffected by simultaneous expression of B5 (Figure 7Ah). Nip3 induced apoptosis was also inhibited significantly by E1B19K (Figure 7i). Similar inhibition of Nip3 induced apoptosis by Bcl-2 in Rat-1 cells was observed at early stage of the apoptotic process.[20] Inhibition of Nip3 induced apoptosis by B5 was weaker than that caused by E1B19K (Figure 7j).

Effects of B5 mutants carrying a deletion in either N-terminal, middle or C-terminal region on the induction of apoptosis by E1A, Nip3 or B5 itself were similarly analyzed by the increase in the sub-G1 population in KB cells (Figure 7B). Among these deletion mutants, B5ΔM and B5ΔC had no significant activity to induce apoptosis as did wild type B5 (Figure 7Bb and c), while B5ΔN lacking the N-terminus increased the sub-G1 population to 11.5% (Figure 7Ba) which is significantly higher than the background value. This increase was repeatedly observed, suggesting that the potential activity of B5 to induce apoptosis is masked by the N-terminal region which has no sequence homology with the corresponding region of Nip3. All the three B5 mutants had no effects on the induction of apoptosis by E1A (Figure 7Bd–f). However, apoptosis induced by Nip3 was significantly inhibited by coexpression with B5ΔN and B5ΔM, but not by B5ΔC (Figure 7Bg–i), suggesting that B5 inhibits apoptosis induced by Nip3 through interaction with Nip3 at the C-terminal region. The extents of apoptosis inhibited by B5ΔN and B5ΔM were about 30% when calculated after subtraction of the background value and were similar to that caused by wild type B5.

## Discussion

In the present study, cellular proteins that interact with adenovirus E1B19K protein were analyzed by isolating the cDNA clones using yeast two hybrid system. Analysis of the structural features of the B5 protein (B5) encoded by one of these clones (Figure 1) revealed that B5 contains the putative BH3 domain in the middle region and the transmembrane domain in the C-terminal region, which is characteristic of the members of the Bcl-2 and E1B19K binding protein families. Through this putative transmembrane region, B5 homodimerizes and heterodimerizes tightly with Nip3, weakly with E1B19K, but not with Bcl-2. The homo- and heterodimerization may occur through hydrophobic interaction of the LXXLL motifs,[25] since Nip3 has this motif and B5 has LXXVL sequence in their transmembrane regions. The C-terminal transmembrane domain is also required to localize B5 to nuclear envelope, endoplasmic reticulum and mitochondria, as does Nip3 and the Bcl-2 family members to these membranaceous structures. B5 has high sequence homology with Nip3 in the middle and C-terminal regions, but has unique sequence in the N-terminus.

The members of the Bcl-2 family are regulators of apoptosis and have function either to induce or to inhibit apoptosis. Most of these members have common conserved regions, designated Bcl-2 homology region, BH1, BH2 and BH3.[10,26,30] E1B19K shares limited sequence homology with Bcl-2 family members just before BH2 and in BH1.[5,6] These homologous sequences are essential for suppression of apoptosis and to cooperate with E1A for transformation of rodent cells. Among these three BH domains, B5 contains only the BH3-like sequence in the middle of the molecule, as do another E1B19K binding proteins, Nip3 and Nbk/Bik.[15,16,20] The BH3 domain has been shown to be sufficient for the interaction of Bax, a member of the Bcl-2 family with E1B19K and Bcl-2.[10] BH3 proteins are therefore supposed to cause cell death by interacting with and antagonizing anti-apoptotic members of the Bcl-2 family through their BH3 domains.[28]

B5 itself has no ability to induce apoptosis, but inhibits apoptosis induced by Nip3, although the inhibition is weaker than that caused by E1B19K. The N-terminal deletion mutant, B5ΔN(Δ1–31), however, acquired the ability to induce apoptosis. Neither B5ΔM(Δ58–96) nor B5ΔC(Δ175–219) acquired this apoptotic ability, suggesting that the N-terminal region, whose aa sequence differs from that of Nip3, masks the potential ability of B5 to induce apoptosis. All the BH3-containing proteins so far characterized, Nip3, Nbk/Bik and Bid[29] are positive regulators of apoptotis, while B5 is a sole member which negatively regulates apoptosis. Similar negative role of the N-terminal region on the induction of apoptosis has been reported with Bim, another BH3 protein containing the C-terminal transmembrane region.[30] Bim has three isoforms, probably generated by alternative splicing, that progressively reduce the length of the N-terminus. All three induce apoptosis, but the shortest Bims has the most potent activity, suggesting that the longer N-termini might act negatively on the ability of the protein to induce apoptosis.

Apoptosis induced in KB cells by Nip3 occurred much stronger than did E1A, whereas B5 inhibited apoptosis induced by Nip3 but not apoptosis induced by E1A. The difference in the potency to induce apoptosis between E1A and Nip3, however, is partly dependent on the transfection period before cell harvest, since the induction of apoptosis by E1A takes much longer than Nip3. Although E1B19K protected apoptosis induced by Nip3 more effectively than that induced by E1A, the protection also depends on the stage of the apoptotic process. Overexpression of Bcl-2 delays the onset of apoptotis induced by Nip3 but the resistance is overcome by a longer period of incubation.[20] The inhibition of Nip3-induced apoptosis by B5 is caused through the C-terminal transmembrane region, since B5ΔC(Δ175–219) lost the ability to inhibit, but B5ΔN(Δ1–31) and B5ΔM(Δ58–96) did not. The Nip3 mutant lacking the C-terminal transmembrane region does not dimerize and localize to mitochondria and loses the ability to induce apoptosis.[20] These results suggest that apoptosis is regulated by the formation of homo- and hetero-dimers in the E1B19K binding BH3 proteins and by the ratio of positive regulators to negative regulators such as B5, in just the same way as the positive and negative regulators of the Bcl-2 family.

We previously showed that topoisomerase IIα is degraded via activation of a component(s) in the ubiquitin-proteasome pathway as one of the targets for E1A induced apoptosis in MA1 cells, a derivative of the KB

cell line.[21,22,31] The present study showed that the level of Nip3 in MA1 cells increased markedly, nearly tenfold, after induction of E1A, while the level of B5 remained almost constant, suggesting that Nip3 might also be involved in executing apoptosis in MA1 cells.

## Materials and Methods

### Cell culture

The cell line MA1 was established from the human epidermoid carcinoma cell line KB by introducing the adenovirus E1A12S cDNA linked to the MMTV-LTR.[21] MA1 cells express E1A12S in response to dexamethasone (dex) and induce apoptosis. KB, MA1 and COS7 cells were cultivated at 37°C in Dulbecco's modified Eagle's minimal essential medium with 10% fetal calf serum (FCS). Dialysed FCS was used for cultivation of MA1 cells.

### Construction of pGAD-MA1 cDNA library

Poly $(A)^+$ RNA was prepared from the mixture of MA1 cells treated with dex for 6, 12, 24, 36 and 48 h according to Okayama *et al*.[32] and reverse transcribed using the oligo(dT) primer with *Xho*I site. The RNA strand of the mRNA/cDNA hybrid was replaced by the corresponding DNA strand by using *E. coli RNAse*H, *E. coli* DNA polymerase I and *E. coli* DNA ligase, and the cDNA was ligated to *Eco*RI linkers after both termini were blunt-ended. After cleaved with *Eco*RI and *Xho*I, the cDNA (100 ng) was ligated to 100 ng of the *Eco*RI/*Sal*I-digested yeast expression plasmid, pGAD424 (Clontech) at 16°C for 48 h. The ligated cDNA was extracted with phenol/chloroform, ultrafiltrated using micron 10 (MILLIPORE) and electrotransformed into *Escherichia coli* DH10B using the *E. coli* Pulser (Bio-Rad) to generate the pGAD-MA1 cDNA library. This library directs the expression of fused proteins between the transactivation domain of Gal 4 and cDNA-encoded polypeptides and replicates autonomously as plasmids in yeasts. The library contained $5.7 \times 10^6$ primary recombinants with an average cDNA size of about 1.5 kb.

### Two hybrid screening

The plasmid pGBT-E1B19K, which directs synthesis of the fused protein between the DNA-binding domain of Gal4 and E1B19K, was introduced into yeast strains HF7C and Y190 and two clones HF7C-E1B19K and Y190-E1B19K were established. These tester strains were transformed with the pGAD-MA1 cDNA library and $his^+$,$leu^+$ transformants grown in S.D. medium containing either 1 mM (for HF7C-E1B19K) or 25 mM (for Y190-E1B19K) 3-AT were scored for $\beta$-galactosidase activity. Each colony was streaked on a square area printed on the nylon filter and incubated by placing the filter on S.D., $His^-$, 3-AT agar medium at 30°C for 2 days. The filter was then dipped in liquid nitrogen and the colonies were frozen and thawed three times. The filter was overlaid onto Whatman 3 MM filters that had been soaked in Z buffer (60 mM Na2HPO4·7H20, 60 mM NaH2PO4·7H20, 10 mM KCl, 1 mM MgSO4·7H20, 50 mM 2-mercaptoethanol, pH 7.0) containing 0.01% X-gal at 30°C for 1–2 h. The positive clones that showed blue color were selected.

### Construction of expression vectors for B5 and its deletion derivatives

B5 cDNA was synthesized by PCR with pGAD-B5 as the template, and the upstream sense primer from codons 1–7 fused to the *Sal*I recognition sequence and the downstream antisense primer from codons 214–219 fused to the *Bam*HI recognition sequence. The PCR product was cleaved with *Sal*I and *Bam*HI and inserted into the *Sal*I-*Bam*HI site of pH$\beta$APr-1[33] to generate pH$\beta$APr-B5. pH$\beta$APr-B5$\Delta$C which encodes B5 lacking the C-terminal sequence from codons 175–219 was constructed by synthesizing B5$\Delta$C cDNA using the same upstream sense primer and the downstream antisense primer from codons 169–174 fused to the *Bam*HI recognition sequence, and similarly inserting into pH$\beta$APr-1. pH$\beta$APr-B5$\Delta$N which encodes B5 lacking the N-terminal sequence from codons 1–31 was constructed by synthesizing B5$\Delta$N cDNA using the upstream sense primer from codons 32–38 fused to the *Sal*I recognition sequence and the downstream primer used for construction of pH$\beta$APr-B5. For construction of pH$\beta$APr-B5$\Delta$M, the N-terminal portion of B5 cDNA was synthesized with the same upstream primer used for synthesis of B5 cDNA and the downstream antisense primer from codons 51–57 fused to the *Eco*RI recognition sequence and inserted into the *Sal*I-*Eco*RI site of pBluescript-$KS^+$ to generate pBluescript-B5N. The C-terminal portion of B5 cDNA was synthesized with the upstream sense primer from codons 97–103 fused to the *Eco*RI recognition sequence and the same downstream primer used for synthesis of B5 cDNA. The PCR product was cleaved with *Eco*RI and *Bam*HI and inserted into the *Eco*RI-*Bam*HI site of pBluescript-B5N. The DNA was then cleaved with *Sal*I and *Bam*HI and the B5$\Delta$M DNA having a deletion between codons 58 and 96 was similarly inserted into pH$\beta$APr-1 to generate pH$\beta$APr-B5$\Delta$M.

The expression vectors for Flag epitope-tagged B5 and its deletion derivatives were constructed using pCXN2 which contains SV40 ori and the multi-cloning site (MCS) downstream of the CMV-1E enhancer and the chicken $\beta$-actin promoter.[34] The B5 cDNA and its derivatives lacking either the N-terminal half ($\Delta$NM) or the C-terminal region ($\Delta$C) with the *Eco*RI recognition sequence at both ends were synthesized by PCR and cloned into the *Eco*RI site within MCS. The vectors, pCXN-FB5, pCXN-FB5$\Delta$C and pCXN-FB5$\Delta$NM express wild type (wt) B5, B5 lacking the C-terminus from codons 188–219 and B5 lacking the N-terminal half from codons 1–119 with the Flag sequence at their 5′ ends, respectively

### Northern blot hybridization

Total cellular RNA was prepared by the AGPC (acid guanidinium thiocyanate-phenol-chloroform) extraction method[35] and poly(A)-containing RNA was prepared by using Oligotex (dT)30.[36] Aliquots of 3 $\mu$g of poly $(A)^+$ RNA were electrophoresed in 1.1% agarose gels in buffer containing 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA and transferred to nylon membrane filters. RNAs were crosslinked by UV (254 mm) irradiation for 5 min. Hybridization was performed first by preincubating the filters in 5 × Denhardt's solution containing 50% formamide, 5 × SSPE (1 × SSPE is 0.18 M, NaCl 10 mM NaH2PO4 pH 7.0, 1 mM EDTA), 0.5% SDS and 100 $\mu$g/ml of denatured salmon sperm DNA at 42°C for 6 h. Hybridization was carried out by adding $10^6$ c.p.m./ml of $^{32}$P-labeled cDNA probes at 42°C for 16 h. The B5 cDNA probe was synthesized by PCR using the upstream sense primer from codons 1–5 and the downstream antisense primer from codons 57–53 in the presence of [$\alpha$-$^{32}$P]dCTP. The cDNA probes for other mRNAs were prepared by labeling the whole cDNAs by using random priming DNA labeling system (Amersham).

### Immunoprecipitation

COS7 cells ($1 \times 10^5$ cells) were washed twice with DMEM and once with PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% NP40) containing 20 units/ml of aprotinin and

2 mM PMSF. The lysate was centrifuged at 8 000 × *g* for 20 min and the supernatant was precleared by incubating with 20 $\mu$l of protein G-Sepharose with rocking followed by a brief centrifugation. The supernatant was incubated with 3 $\mu$g of anti-Flag antibody M5 at 4°C for 1 h and gently rocked after addition of 20 $\mu$l protein G-Sepharose at 4°C for 1 h. The immune complex was precipitated by centrifugation at 12 000 × *g* for 20 s and washed eight times with 0.5 ml of wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 20 units/ml of aprotinin and 2 mM PMSF.

### Western blotting

Aliquots of COS7 cell lysates or its immunoprecipitates were electrophoresed on 12% polyacrylamide gels and proteins were transferred to Immobilon filters (Millipore). The filters were preincubated in immunoblotting diluent solution (5% skim milk [Difco] and 0.1% Tween20 in PBS) at room temperature for 1 h and incubated with either anti-Flag antibody or anti HA-antibody at appropriate dilutions as indicated in the Figure legends at room temperature for 1 h. The filters were washed three times in PBS containing 0.1% Tween20 and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG at a 1 : 200 dilution. Immune complexes were detected by ECL according to the manufacturer's protocol (Amersham Corp.).

### Immunofluorescence

KB and COS7 cells cultivated on a LAb-Tek Tissue culture Chamber/slide (Miles Scientific) were transfected with pCXN-FB5, pCXN-FB5ΔC or pCXN-FB5ΔNM together with pH$\beta$APr-E1B19k and fixed in freshly prepared aldehyde solution (4% [vol/vol] paraformaldehyde in PBS) at room temperature for 10 min. The fixed cells were washed in PBS, permeabilized in 0.1% Triton X-100 in PBS for 5 min and washed in PBS. To minimize nonspecific binding of antibodies, the cells were preincubated in 0.2 M Tris-glycine, pH 7.4 for 1 h at room temperature. The cells were covered with anti-Flag mouse monoclonal antibody M5 and anti-E1B19K rabbit polyclonal antibody at 1 : 250 dilutions and incubated at room temperature for 30 min. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG goat antibody at a 1 : 2000 dilution and Texas Red-conjugated anti-rabbit IgG donkey antibody at a 1 : 500 dilution at room temperature for 30 min. In all the cases, the cells were washed extensively in Tris-buffered saline and mounted in 87% glycerol (Merck) containing 2.5% 1,4-diazabicyclo [2,2,2] octane (Sigma).

### Flowcytometric Analysis

Sparse cultures of KB cells in 3.5 cm-dishes were transfected with combinations of the expression vectors (each 0.3 $\mu$g) pH$\beta$APr-B5, pH$\beta$APr-Nip3, pH$\beta$APr-E1A12S, and pH$\beta$APr-E1B19K together with 0.1 $\mu$g of pCMVCD20[37] by lipofection using LipofectAMINE PLUS™ transfection reagent (GIBCO-BRL). At 20 h post transfection, the cells were stained with FITC-labeled anti-CD20 antibody (B-B6, Biosource International). A set of the cells was transfected with control empty vector pH$\beta$APr-1 alone and similarly stained with anti-CD20 antibody to determine the background. After staining, the cells were fixed with 4% formaldehyde, treated in 0.1% Triton X-100 briefly to permeabilize the cells, and then stained with propidium iodide (PI) containing 1300 Kunitz unit $ml^{-1}$ RNase A. The cells were analyzed for fluorescence intensity of FITC and PI by using a FACS-Calibour Flow-Cytometer and the cell quest program version 1.2 (Becton-Dickinson). The cell population that showed the fluorescence intensity over this background was gated and analyzed for cell cycle distribution.

## Acknowledgements

We thank Dr. G. Chinnadurai for providing E1B19K mutant 90-6, Dr. E. White for E1B19K mutant pm51, Dr. S. Hashimoto for anti-E1B19K polyclonal antibody, Dr. J. Miyazaki for expression vector pCXN2 and Dr. E. Harlow for the CD20 expression vector.

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