

GFP를 이용하여 in-vivo에서 추적한 Bad와 Bcl-XL의 Mitochondria 이동

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= Abstract =

Bad Translocation to Mitochondria with Bcl-XL Traced in-vivo by Using GFP

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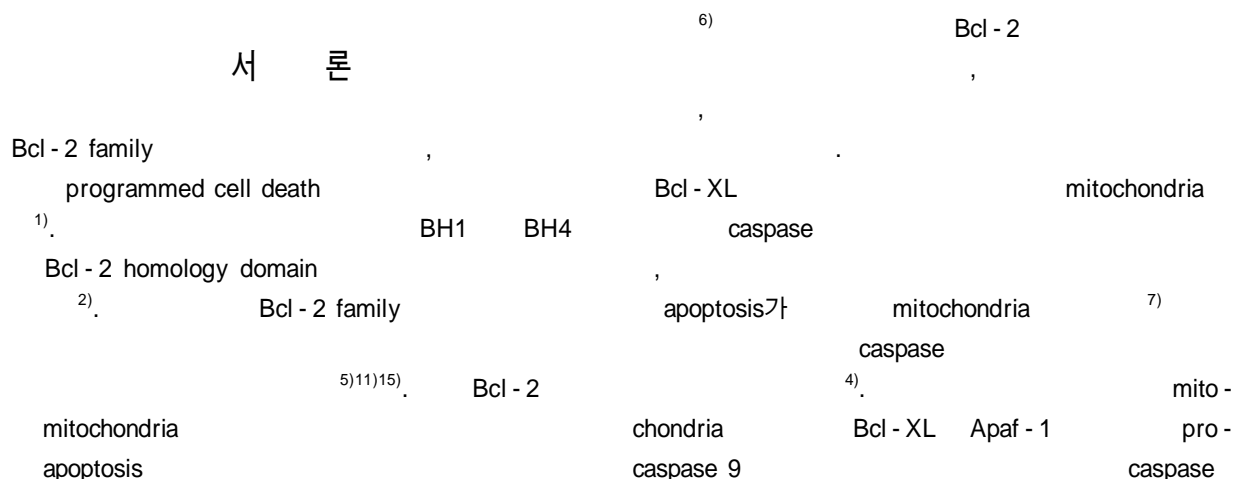
Objectives : The subcellular locations of Bad, Bid, Bax and Bcl - XL change during apoptosis and this change is important for the regulation of cell death. The purpose this study was to elucidate binding of Bad with Bcl - XL in vivo

Methods : We made Bad with Green Fluorescent Protein(GFP) using PCR method. We transfected and overexpressed GFP - Bad with or without Bcl - XL cotransfection in living COS - 7 cell.

Results : Bad and Bcl - XL bind one another in healthy living cells and this association controlled mitochondrial docking. In the absence of Bad - XL, Bad was mainly cytosolic and partially bound to mitochondria. Upon coexpression of Bad and Bcl - XL, most of Bad translocated to mitochondria.

These should suggest that Bad binds to the mitochondrial and cytoplasmic forms of Bcl - XL and Bad bound to cytoplasmic Bcl - XL translocates to mitochondria. These in vivo findings confirm that Bad make a complexes with Bcl - XL and cause mitochondrial translocation of Bad - Bcl - XL complex.

KEY WORDS : Apoptosis · Bad · Bcl - XL · Green fluorescent protein.



GFP *in-vivo* Bad Bcl-XL Mitochondria

가 ¹⁰⁾¹²⁾ Bcl - XL Bad
 Bcl - XL ¹⁷⁾¹⁸⁾,
 Bad phosphorylation
³⁾¹⁹⁾ Bcl - XL
⁸⁾⁹⁾ 가
 가 Bad Bcl - XL
 Green fluorescence protein
 (GFP) Bad Bcl - XL
 Bad

재료 및 방법

1. 재 료

Cos - 7 cell ATCC
 primer GIBCO BRL
 pcDNA3 mammalian expres -
 sion vectors Invitrogen(Carlsbad, CA)
 C3 - EGFP plasmid Clontech Laboratories Inc.
 (Palo Alto, CA) Lipofectamine Life
 Technologies(GIBCO BRL, Gaithersburg, MD)
 polyclonal anti - N - terminal Bad antibody sc -
 941 Santa Cruz Inc.(Santa Cruz, CA) anti -
 phospho Bad polyclonal antibody Et - Te Hsu
 (NIH, MD, USA) Donkey anti - rabbit
 immunoglobulin peroxidase conjugates ECL Western
 blotting detection kit Amersham Corp.(Arlington Hei -
 ghths, IL) Sigma Chemical Co.
 (St. Louis, MO)

2. 방 법

1) Bad plasmid cloning과 mutagenesis

Bad HA - tagged Mouse Bad cDNA(Michael Green -
 berg (UCLA, CA)가) PCR C3 -
 EGFP plasmid(Clontech Laboratories, Inc., Palo Alto,
 CA) EcoRI BamHI pcDNA3
 mammalian expression vector(Invitrogen, Carlsbad,
 CA) EcoRI cloning Bcl -
 XL PCR C3 - EGFP plasmid pcDNA3 EcoRI

2) Cos-7 세포에 Bad를 일시적으로 transfection
 Cos - 7 monkey kidney epithelial cell confo -
 cal microscopy 4.3cm² chamber slides(Lab -
 Tek chambered coverglass system ; Nalge Nunc Inc.,
 Naperville, IL) DNA transfection
 0.5ug DNA , 2 DNA
 cotransfection 1 : 4 (C3 - EGFP - Bad
 construct : pcDNA3 - Bcl - XL Bax construct) 3ul
 LipofectAMINE()

3. Western blotting에 의한 Bad의 세포내 위치확인

Western blotting Bad
⁸⁾
 cotransfection 3 (100mm)
 Cos - 7 80 90% confluency
 4ug C3 - EGFP - Bad 16ug pcDNA3 - Bcl - XL,
 pcDNA3 vector LipofectAMINE(24ul/plate)
 transfection Cotransfection 36
 PBS
 Dounce homogenizer homogenize
 Beckman TLA 120.2 rotor 130,000 × g
 Bad
 SDS lysis buffer 가 polyclonal anti -
 N - terminal Bad antibody sc - 941 western
 blotting

4. Confocal microscopy

4.3cm² chamber slide Cos - 7 DNA
 transfection 16 24 confocal microscope
 mitochondria mi -
 tochondria - specific dye(Mitotracker Red CMXRos ;
 Molecular Probes Inc., Eugene, OR) 20ng/m가
 20 confocal microscope(a model LSM
 410 confocal, Carl Zeiss, Thornwood, NY)
 600 Chamber slide 35
 37 air stream incubator GFP
 580nm , mitotrac -
 ker 420nm sta -
 uro - spo - rine 가 0.5uM
 , confocal microscpe 5 10 4 6

5. 세포생존율
Bad

mouse fibroblast L929
6 well plates
C3 - EGFP vector the pcDNA3 vector, C3 -
EGFP - Bad pcDNA3 vector, C3 - EGFP vec -
tor pcDNA3 - Bad LipofectAMINE cot -
ransfection . 36
sporine 0.1uM 가
GFP
stauro -
가 24

결 과

1. Cos-7 세포주에서 과발현된 GFP-Bad의 세포내 위치

Cos - 7 GFP - Bad
, mitochondria

Mitotracker

Cos - 7 GFP - Bad가 mitoc -
hondria Mitotracker

(Fig. 1A, B, C). Bad가 staurosporine
가 apoptosis Bad
가 mitochondria Mitotracker
().

2. GFP-Bcl-XL의 세포 내 위치

Bcl - XL mitochondria
GFP
mitochondria
34). GFP Bcl - XL
hondria (Fig. 1D, E, F).
mitochondria

transfection

가

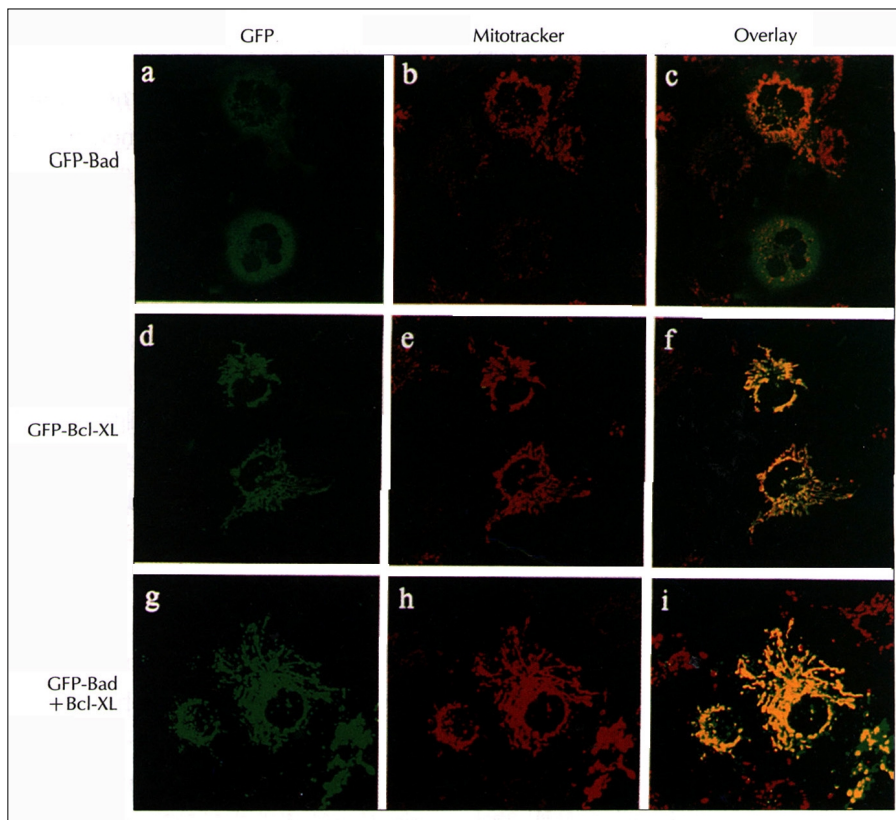


Fig. 1. Bcl-XL alters Bad location in Cos-7 cells. Transiently transfected Cos-7 cells were treated with 20ng/ml Mitotracker Red CMXRos to stain mitochondria and then examined with laser fluorescence confocal microscopy. Each field was independently observed at 480 nm wavelength for GFP (A, D, and G) and at 560 nm for Mitotracker Red CMXRos (B, E, and H) and the two images were overlaid (C, F, and I). GFP-Bad translocates to the mitochondria when cotransfected with Bcl-XL.

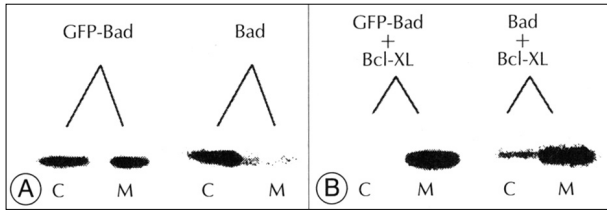


Fig. 2. Comparison of Bad with GFP-Bad in the subcellular location in Cos-7 cells. A : GFP-Bad shows more in the supernatant than pcDNA3-Bad does. B : Upon cotransfection with Bcl-XL(1 : 4 ratio) both GFP-Bad and Bad move into the membrane pellet(C : cytosolic fraction, M : membrane fraction).

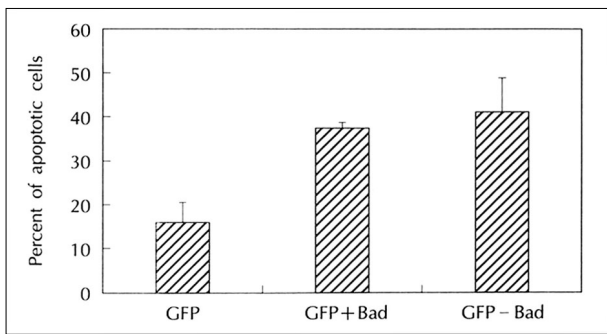


Fig. 3. Comparison of Bad with GFP-Bad bioactivity. Both GFP-Bad and Bad show the same degree of cell death stimulation after 24hours exposure to 0.1uM staurosporine comparing to control(GFP only) L929 cells.

3. pcDNA3-Bcl-XL과 동시에 transfection된 GFP-Bad의 세포 내 위치
 Bcl - XL pcDNA3 , Bad C3 - EGFP vector
 transfection confocal mi -
 croscopy GFP - Bcl - XL Mitotracker가
 mitochondria GFP - Bad Mitot -
 racker가 mitochondria
 (Fig. 1G, H, I).

4. GFP-Bad와 pcDNA3-Bad의 western blotting과 생존율
 GFP construct Bad
 GFP - Bad pcDNA3 - Bad pcDNA3
 vector cotransfection western blotting
 pcDNA3 - Bad , GFP -
 Bad pcDNA3 - Bad mitochondria
 (Fig. 2A). GFP - Bad pcDNA3 - Bad
 Bcl - XL cotransfection GFP - Bad
 pcDNA3 - Bad
 (Fig. 2B).

GFP vector transfection

GFP vector pcDNA3 - Bad transfection
 , GFP - Bad transfection
 (Fig. 3).

GFP construct Bad
 가 GFP
 Bad Bad

고 찰

Bcl - 2 family BH1 - 4 4 domain 가
 , domain Bcl - 2, Bcl - XL, E1b - 19k
 anti - apoptotic , Bax, Bok pro - apoptotic ,
 Bad, Bid, Bik BH3 1).
 Bax - Bcl - 2, Bax - Bcl - XL, Bad -
 Bcl - XL, Bim - Bcl - 2, , Bik - Bcl - XL Bcl -
 2 fa - mily hetero - dimerization Bax,
 Bcl - 2, Bcl - XL homo - dimerization
 , Bcl - 2 - calcineurin¹⁴⁾, Bcl - 2 - NFAT
 (Nuclear factor of activated T cells)¹³⁾, Bcl - XL -
 Apaf - 1¹²⁾ Bcl - 2 family het -
 ero - dimerization
 Bax, Bcl - 2, Bcl - XL transmem - brane do -
 main 가 mitochondria rere -
 cptor docking
 , docking 가
 , docking mitochon - dria
 voltage - dependent anion channel (VDAC)
 water channel mitoch - ondria
 cytochome C
 6).
 dimerization theory in - vitro immu -
 noprecipitation yeast hybridization assay
 , Hsun ⁹⁾ NP - 40 triton X - 100,
 W - 1 detergent dim -
 erization 가 , detergent
 hetero - dimerization
 dimerization
 가 . in - vivo Bad -
 Bcl - XL hetero - dimerization in - vivo
 . PCR GFP
 Bad N - terminal lipofectamine
 transfection

confocal microscope

western blot

7)16)

GFP Bad DNA Bad

, GFP - Bad transfection cyto -

plasm , Bcl - XL GFP - Bad trans -

fection mitochondria , frac -

tional western . Hsu YT 7)

western Bcl - XL

50%가 mitochondria , 50%

가

GFP - Bcl - XL 가 confocal microscopy Cos -

7 mitochondria ,

GFP Bcl - XL 3 가

Bcl - XL mitochondria docking

. Bad Bcl - XL transfection

Bad가 mitochondria Bad가 mito -

chondria Bcl - XL ,

Bcl - XL mitochondria

Bcl - XL 가 Bad

mitochondria docking 가

membrane domain Bcl - XL Bad transme -

Bad가 mitochondria

() Bad가

Bcl - XL dimerization mitochondria docking

. , Bad Bcl - XL

transfection Bad Bcl - XL

dimerization mitochondria

docking

Bad Bcl - XL binding mitochon -

dria docking

. Bad pro - apo -

ptotic Bcl - XL dimerization mitochondria

docking

가

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- : 2000 3 7
- : 442 - 749 5

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