이학박사학위논문

c-Cbl 엑손 건너뛰기가 αPix 에 의해 매개되는 뇌종양 세포의 이동과 침투에 미치는 영향에 대한 연구

Studies on the effect of c-Cbl exon skipping on αPix-mediated cell migration and invasion of glioma cells

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생명과학부

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Studies on the effect of c-Cbl exon skipping on αPix-mediated cell migration and invasion of glioma cells

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By

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ABSTRACT

c-Cbl, a RING-type ubiquitin E3 ligase, down-regulates receptor tyrosine kinases, including EGF receptor, and inhibits cell proliferation, migration, and invasion. Moreover, mutations of c-Cbl are frequently found in patients with myeloid neoplasm. Therefore, c-Cbl is known as a tumor suppressor. αPix, a potent mediator of cell migration, is highly expressed only in proliferative and mobile cells, including immune cells, and is up-regulated in certain invasive tumors, such as glioblastoma multiforme (GBM). Here, I showed that a part of the RING domain in c-Cbl is skipped in rat C6 and human A172 glioma cells and brain tissues of several glioblastoma patients. Specifically, these cells and tissues generated c-Cbl that lacks the amino acid sequences corresponding to exon-9 or both exon-9 and exon-10, termed type I and type II exon skipping, respectively. Significantly, both types of the exon skipping prevented c-Cbl-mediated ubiquitination and proteasomal degradation of αPix, resulting in

dramatic accumulation of αPix. Moreover, the c-Cbl exon skipping promoted EGF signaling and αPix-mediated cell migration and invasion. However, analysis of the c-Cbl genomic DNA sequence revealed that no putative splice site mutation exists between exon-8 and exon-11. Furthermore, both types of the exon skipping could be seen only when C6 and A172 cells were grown to confluent state (i.e., in contact inhibition) or under hypoxia conditions, as analyzed by using a mini-gene harboring the genomic DNA from exon-7 to exon-11. These results suggest that unknown defect of *trans*-element(s) in C6 and A172 cells, likely also in the tissues from glioma patients, is responsible for the abnormal exon skipping, which in turn allows the glioma cells to overcome contact inhibition and hypoxic condition. In summary, these findings indicate that the c-Cbl exon skipping contributes to human glioma and its malignant behavior.

Keywords: c-Cbl, αPix, glioma, exon skipping, confluency, hypoxia

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αPix is up-regulated in C6 and A172 glioma cell lines.

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c-Cbl proteins generated by exon skipping are inactivated and destabilized.

c-Cbl exon skipping up-regulates EGF signal.

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BACKGROUND

1. c-Cbl

Ubiquitin is a small protein modifier, which consists of 76-amino acid. The C-terminal glycine residue of ubiquitin is chemically cross-linked to ε-amino group of substrate lysine residues. The enzymatic cascade utilizes ubiquitin activating E1 enzyme, ubiquitin conjugating E2 enzyme, and ubiquitin E3 ligase as ubiquitin-transfer machinery (Figure 1) (Hershko and Ciechanover, 1998; Pickart and Fushman, 2004). Several types of ubiquitination have been reported, which include mono-ubiquitination, poly-ubiquitination, linear poly-ubiquitination, and branched poly-ubiquitination. Of these, poly-ubiquitination can be subdivided by the isopeptide bonds formed with different lysine residues in ubiquitin (i.e., the use of Lys48, Lys63, and Lys29 etc.) and Lys48-linked poly-ubiquitination recruits the target proteins to the 26S proteasome for degradation (Kulathu and Komander, 2012).

Ubiquitin-proteasome system (UPS) plays essential role in the control of numerous important cellular processes, including recycling of proteins, signal transduction, endocytosis, cell migration, and transcription coupled repair. Contrary to lysosomal degradation, UPS is not random or nonspecific process, but precisely controlled and target-specific process. These characteristic regulatory features of UPS are typically maintained by regulation of E3 ligases owing to their specificity to substrate proteins. Post-translational modification of E3 or substrate, transcriptional and translational regulation of E3, regulation of E3 inhibitor, and alternative splicing of E3 are well known cellular regulation of UPS (Kee and Huibregtse 2007; Merlet et al., 2009; Shimizu and Hupp, 2003).

In the late 1980s, c-Cbl was identified as cellular homologue of v-Cbl oncogene, which is the transforming gene of the murine Cas NS-1 retrovirus (Langdon et al., 1989). After a decade later, the RING domain of c-Cbl was identified, and E2 binding and ubiquitin transfer activity of c-Cbl were

discovered (Levkowitz et al., 1998). It is now known that c-Cbl is a RING type ubiquitin E3 ligase, which ubiquitinates various oncoproteins, including receptor tyrosine kinases (RTKs), focal adhesion complex proteins, and proteins related with growth factor signal. Thus, c-Cbl is known as a tumor suppressor protein. c-Cbl consists of four distinctive domains: the tyrosine kinase binding domain (TKB), the RING domain, the proline-rich domain, and the ubiquitin associated domain/leucine zipper domain (UBA/LZ) (Figure 2). The TKB domain mediates binding to phosphotyrosine residues of RTKs, which is important for substrate recognition. The RING domain is E2-ubiquitin complex binding domain, which is essential for ubiquitin transfer. The proline-rich domain is involved in the adaptor functions of c-Cbl by mediating binding of several Src-homology-3 (SH3)-domain-containing proteins. Lastly, the C-terminal UBA/LZ domain mediates oligomerization of c-Cbl and ubiquitin binding (Schmidt and Dikic, 2005).

Upon growth factor binding to receptors, downstream kinases phosphorylate c-Cbl at several tyrosine residues, which activates the enzymatic activity of c-Cbl. Of these residues, Tyr368 and Tyr371 play a key role in activation of c-Cbl. Crystal structure of c-Cbl revealed that phosphorylation of Tyr368 and Tyr371, which are embedded in the middle of short peptide region between TKB and RING, lead to conformational change from closed state to open state. As shown in Figure 3, distance between E2-ubiquitin complex and substrate is shortened, and ubiquitin transfer is easily conducted in the open state (Dou et al., 2013; Zheng et al., 2000).

Mutations of c-Cbl are frequently found in myeloproliferative diseases, developmental disorders, Noonan syndrome, and lung cancer (Ogawa et al, 2010; Niemeyer et al., 2010; Martinelli et al., 2010; Tan et al., 2010). Numerous types of c-Cbl mutation have been reported, including missense mutations, deletions, insertions, and splice site mutations. Most of mutations were found in the middle of linker and the RING domain, which are essential

for activation of E3 ligase. Exon skipping mutations of c-Cbl are also frequently found, and exon-8 and exon-9 are the most frequently skipped exons, which encode the linker and RING domains. Moreover, several c-Cbl mutants dominant negatively inhibit wild type c-Cbl, indicating that heterozygous mutations can sufficiently develop disorder (Sanada et al., 2009). In this respect, c-Cbl is one of prominent E3 ligases that are associated with human diseases.

Figure 1. Ubiquitin Proteasome System (UPS)

Ubiquitination of target proteins comprises three cascade enzymatic steps. Ubiquitin activating enzyme (E1) activates ubiquitin at the expense of ATP, and a thiolester bond is formed between ubiquitin and E1. Activated ubiquitin is transferred to ubiquitin conjugating enzyme (E2), forming a thiolester bond between E2 and ubiquitin. Binding of specific substrate to ubiquitin ligase (E3) recruits activated E2, results in intermediate complex comprising the target protein, E3, and ubiquitin~E2. Ubiquitin is then ligated to a lysine residue of the target protein. This process continues to generate poly-ubiquitin chain(s), which subsequently recognized by 26S proteasome complex for degradation of the target protein to small peptides. Deubiquitinating enzyme (DUB) removes ubiquitins from the target protein for stabilization.

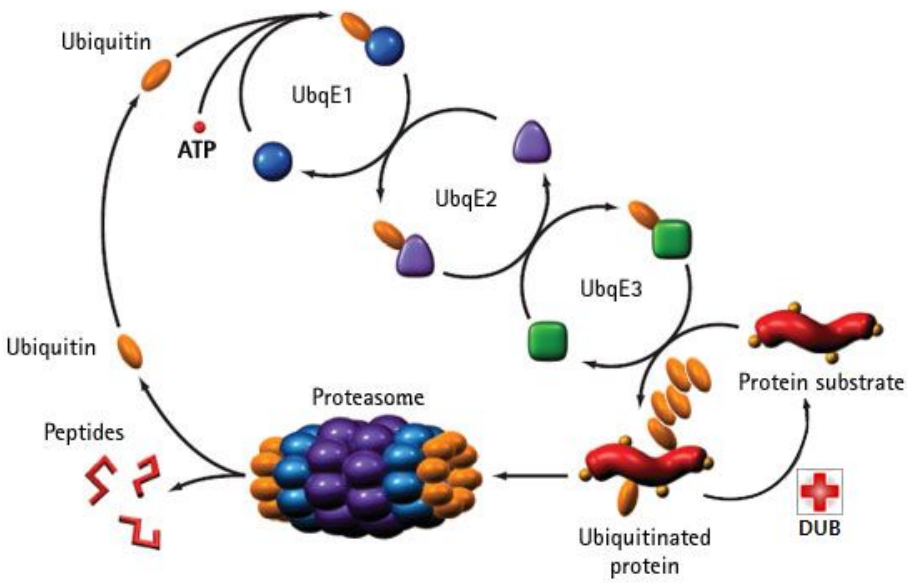

Ubiquitin
UbqE1
ATP
UbqE2
UbqE3
Ubiquitin
Protein substrate
Proteasome
Peptides
DUB
Ubiquitinated
protein

Figure 2. Primary structure of c-Cbl

Schematic representation of c-Cbl primary structure. The N-terminal tyrosine-kinase-binding (TKB) domain mediates its binding to phosphotyrosine residues of RTKs. The RING domain is important for the transfer of ubiquitin from E2 enzymes to the substrates. The region between the TKB and RING domains is referred to as the linker, and is often deleted or mutated in oncogenic c-Cbl variants. The proline-rich domain is involved in the adaptor functions of c-Cbl by mediating its binding of several Src-homology-3 (SH3)-domain-containing proteins. Lastly, the C-terminal UBA/LZ domain has a role in c-Cbl oligomerization and ubiquitin binding. Note that phosphotyrosine and phosphoserine residues are important regulatory sites within c-Cbl.

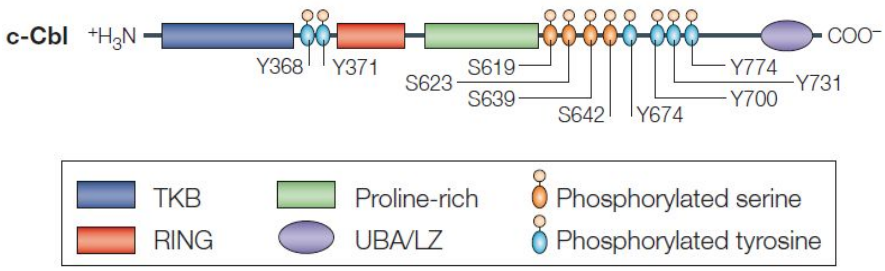
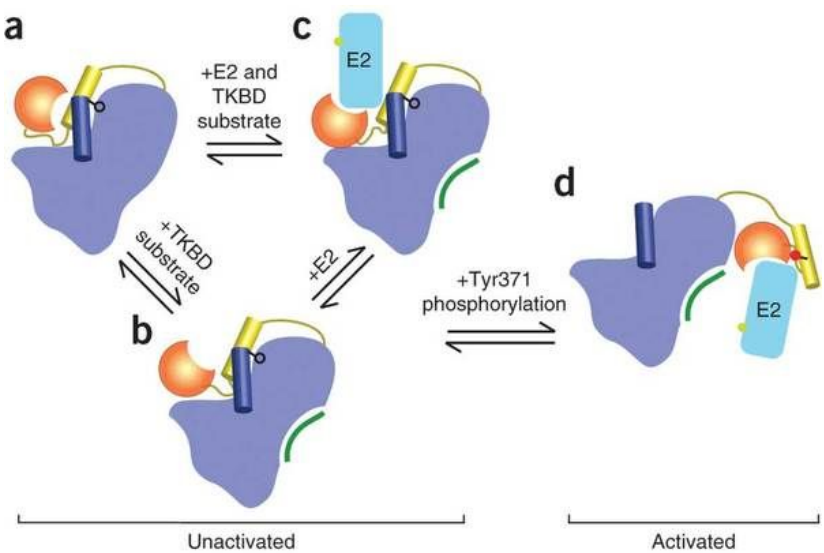

c-Cbl
+H3N
COO−
Y368
Y371
S623
S619
S639
S642
Y674
Y774
Y731
Y700
TKB
RING
Proline-rich
UBA/LZ
Phosphorylated serine
Phosphorylated tyrosine

Figure 3. Activation mechanism of c-Cbl

(**a**) In the absence of E2, c-Cbl adopts a closed conformation, in which the E2-binding surface of RING associates with TKBD. (**b**) TKBD substrate binding induces partial RING opening. (**c**) E2 binding causes the RING domain to adopt an open conformation. TKBD competes with E2 for RING binding, which reduces E2 affinity and E3 activity. In the inactivated state, Tyr371 (black ball-and-stick) secures LH to TKBD and limits the RING domain rotation to a region distal from the TKBD substrate-binding site. (**d**) pTyr371 (red ball-and-stick) activates c-Cbl by releasing LH from TKBD, thereby abolishing auto-inhibition, altering LH-RING-E2 interactions, and promoting marked LHR conformational changes that bring the RING domain and E2 into proximity of the substrate.


a
c
E2
+E2 and
TKBD
substrate
+TKBD
substrate
+E2
d
+Tyr371
phosphorylation
E2
b
Unactivated
Activated

2. αPix

Cell adhesion and migration is key feature of cancer progression. Malignant cancer cells lose their polarity and are detached from original site, and detached cells undergo migration toward growth factor and chemokine. Cell migration is a step-by-step procedure, which is proceeded via focal adhesion. Focal adhesion is considered as “feet” of cells, whose appearance resembles the suckers of octopus (Gordon et al, 2005). During cells move toward growth factors, new focal adhesion is formed at the end of leading edge, and downstream signal transduced from focal adhesion organizes actin filaments into bundle. Actin bundles draw cell bodies to focal adhesion, and cells migrate toward the leading edge. Therefore, focal adhesion functions as mechanical footstep and signaling platform of migrating cells (Rosenberger and Kutsche, 2006).

Focal adhesion complex is composed of four interacting module: ECM binding module, actin linking module, actin polymerizing module, and

adhesion signaling module (Nakada et al., 2007). Of these, adhesion signaling module is dynamic machinery, which determines the direction and rate of migration. Rho family of GTPases transduces adhesion signal by activating Pak family of kinases. Activated Pak phosphorylates downstream target proteins and finally organizes actin bundle. Three GTPases known to involve in cell migration are Rac1, Cdc42, and RhoA. Of these, Rac1 and Cdc42 determine the direction of migration by forming filopodia and lamellipodia at the early stage of cell migration, while RhoA determines the migration rate by constructing stress fibers (Ridley, 2006).

Pix proteins (Pak interacting exchange factor, also known as COOL and Arhgef) are guanine nucleotide exchange factors (GEFs), which exchange GDP to GTP in the GTP binding pocket of Rac1 and Cdc42. Pix proteins also function as signal scaffolds by linking GTPases to Paks. Two isoforms of Pix proteins exist in mammalian cells: one is αPix and the other is βPix. Pix proteins consist of six distinctive domains, as shown in Figure 4. Dbl

homology domain (DH) is the GTPase binding domain and Src homology 3 domain (SH3) is the Pak interacting domain, both of which play an important role in adhesion signal transduction. GBD is the GIT (also known as p95-APP) binding domain, which is required for endosome trafficking of Pix proteins (Scott and Steen, 2008). As shown in Figure 5, Pix proteins are recruited to the leading edge of membrane protrusion via endosome trafficking, forming Pix-Pak-GIT complex. Coiled coil (CC) domain is oligomerization domain, which is required for scaffold function of Pix proteins. Lastly, calponin homology (CH) domain is present only in the αPix. Partial deletion of CH domain was found in the genetic disorder, X-linked mental retardation, however, the precise function of CH domain is not known (Kutsche et al., 2000).

αPix is expressed only in the highly mobile cells, including immune cells, microvascular cells, and growing fetal neurons (Singh et al., 2013; Santiago-Medina et al., 2012; Missy et al., 2008). Unlike αPix, βPix is ubiquitously

expressed in every kind of tissues. Recently, αPix has been shown to be up-regulated in the malignant AGS gastric cancer cells and GBM patients (Yokota et al., 2006; Baek et al., 2007). The precise functional relationship between αPix and βPix remains to be uncovered. However, it is known that that αPix is expressed only in rapidly migrating cells, implicating its role in invasiveness of malignant cancer cells, particularly when the expression of αPix is abnormally up-regulated.

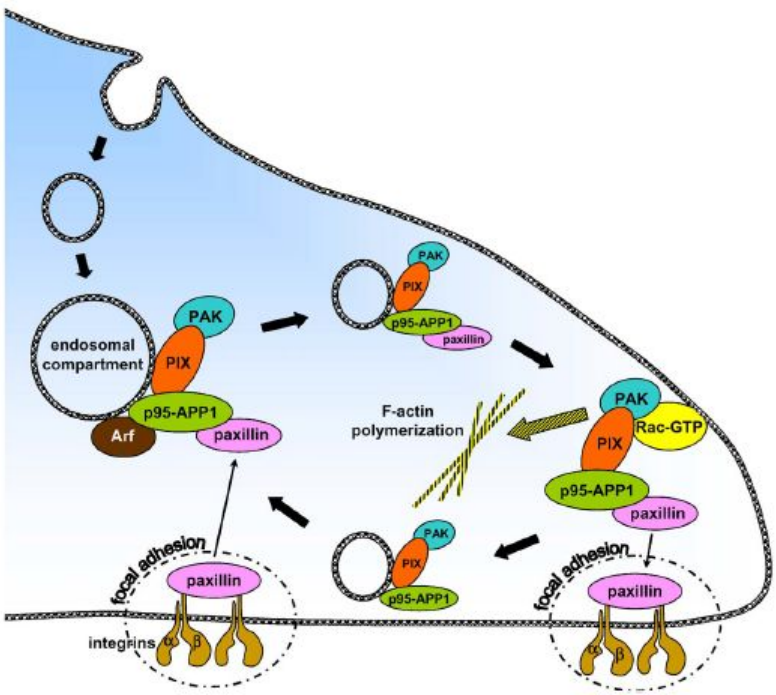
Figure 4. Primary structure of Pix proteins

PIX proteins contain a src homology 3 (SH3), a dbl homology (DH), a GIT-binding domain (GBD), and a coiled-coil (CC) domain. Note that the calponin homology (CH) domain is present only in the αPix.


CH
SH3
DH
GBD
CC
αPix
βPix

Figure 5. Function of Pix proteins

Model for the role of the p95-APP1/GIT-PIX-PAK complex in trafficking between the endosomal compartment and the plasma membrane. PIX interacts with both p95-APP1 and PAK. The ankyrin repeats of p95-APP1 as well as PIX may induce recruitment of the complex to the endosomal compartment. In addition, the Arf-GAP domain of p95-APP1 seems to regulate Arf6 function and thereby may mediate vesicle budding from the endosomal compartment. Re-localization of the focal adhesion protein paxillin from focal adhesions to the p95-APP1-PIX-PAK complex is mediated by its association with the C-terminal portion of p95-APP1. Subsequently, activated PAK could mediate actin reorganization that is required for productive protrusive activity and paxillin could be released to participate in focal adhesion formation. When focal adhesions break down in a moving cell, the p95-APP1-PIX-PAK complex may move back to the endosomal compartment.


endosomal compartment
PAK
PIX
p95-APP1
Arf
paxillin
PAK
PIX
p95-APP1
paxillin
F-actin polymerization
PAK
Rac-GTP
PIX
p95-APP1
paxillin
PAK
PIX
p95-APP1
focal adhesion
paxillin
integrins
α
β
focal adhesion
paxillin
α
β

3. Purpose of this work

Prognosis of GBM patients is poor even after surgical removal and chemotherapy, because of invasive behavior of glioma cells. Complete surgical removal of glioma cells is impossible, because boundary between glioma cells and normal brain tissue is not clear (Behin et al., 2003; Wen and Kesari, 2008). Moreover, chemotherapy is also difficult, because blood brain barrier blocks the delivery of chemicals. Although a great deal of efforts has been made for the search of appropriate drug against glioma, temozolomide (TMZ) is the only available therapeutic drug. However, the development of glioma cells resistant to TMZ is another obstacle in glioma therapy. Moreover, TMZ resistant glioma cells show more severe invasiveness (Spinelli, 2012). Numerous genes related with invasiveness of glioma have been reported. However, the molecular mechanism of glioma invasiveness remains to be solved.

In this thesis, therefore, I aimed to identify the protein factor that is involved in glioma invasiveness, to determine the molecular mechanism how the malignant behavior of glioma cells is regulated, and to uncover the causatives that contribute to the development of the brain cancer.

INTRODUCTION

Migration and invasion are malignant behaviors of cancer cells (Bravo-Cordero et al., 2012). Glioblastoma multiforme (GBM) is the most invasive and aggressive human brain tumor. Disease-free survival of GBM patients is poor even after surgical removal, radiotherapy, and chemotherapy, because of the malignant behavior of glioma cells (Behin et al., 2003; Wen and Kesari, 2008). Therefore, unlike for common types of solid cancer, current experimental therapies for GBM are mainly focused on inhibition of invasion (Chakravarti and Palanichamy, 2008; Mischel and Cloughesy, 2003; Fine, 2014; Lu et al., 2012). Numerous proteins are involved in invasiveness of glioma cells. They include focal adhesion complex proteins, such as Pix, integrin, and paxillin, and receptor tyrosine kinases (RTKs), including EGFR and c-Met (Nakada et al., 2007).

The Pix proteins (also called COOL or Arhgef) are guanine nucleotide

exchange factors (GEF) that activate the Rho family of small GTPases. Of these, αPix (COOL-2) activates Cdc42 and Rac1 by exchanging GDP to GTP (Baird et al., 2005; Baird et al., 2006; Manser et al., 1998; Obermeier et al., 1998). Upon ligand binding to integrin, αPix functions in the activation of Pak kinase by forming a complex with GIT and Pak at the focal adhesion (Zhao et al., 2000). αPix is also involved in cell spreading, direction of migration, angiogenesis, and dendrite outgrowth of fetal neurons (Obermeier et al., 1998; Lucanic and Cheng, 2008; Meseke et al., 2013; Singh et al., 2013; Mazaki et al., 2006; Santiago-Medina et al., 2012). Consistently, αPix could be detected only in highly motile cells, including immune cells, microvascular cells, and growing fetal neurons, unlike βPix (COOL-1) that is ubiquitously expressed in all human tissues (Singh et al., 2013; Santiago-Medina et al., 2012; Missy et al., 2008). Moreover, the protein level of αPix is markedly up-regulated in AGS gastric cancer cells and GBM patient tissues (Yokota et al., 2006; Baek et al., 2007). These findings suggest that up-regulation of αPix in glioma cells might

be responsible for their malignant behavior. However, underlying mechanism(s) for αPix up-regulation in the cancer cells and tissues remained unknown.

c-Cbl, a RING type E3 ubiquitin ligase, down-regulates proteins that are related with cell growth and migration, such as paxillin, FAK, and EGFR (Schmidt and Dikic, 2005; Tan et al., 2010; Truitt et al., 2010; Huang, 2010; Rafiq et al., 2011). Moreover, numerous mutations in c-Cbl have been found frequently in myelo-proliferative diseases, including point mutations, deletions, insertions, and splice site mutations, implicating the role of c-Cbl as a tumor suppressor (Abbas et al., 2008; Martinelli et al., 2010; McKeller et al., 2009; Niemeyer etal., 2010; Reindl et al., 2009). Significantly, βPix interacts with c-Cbl, and prevents c-Cbl-mediated down-regulation of EGFR, thus allowing EGF-mediated signal transduction (Feng et al., 2004; Flanders et al., 2003; Jozic et al., 2005; Schmidt et al., 2006; Wu et al., 2003). However, it remained unknown whether αPix interacts with c-Cbl.

Most of c-Cbl mutations were found in the region between exon-8 and exon-10, which encodes the linker and RING domains of c-Cbl. RING domain is E2 ubiquitin-conjugating enzyme binding site, which is essential for transferring ubiquitin moieties to substrates (Abbas et al., 2008; Reindl et al., 2009; Dikic and Schmidt, 2007). The linker sequence is essential region for activation of c-Cbl. Upon ligand activation by growth factors, two tyrosine residues within linker region are phosphorylated by src-family kinases, and c-Cbl undergoes conformational change from closed state to open state. In open state, the distance between E2-ubiquitin complex and substrate is shortened, and E2 can easily transfer ubiquitin to substrates (Dou et al., 2013; Zheng et al., 2000). Numerous papers reported deletion mutation c-Cbl in myeloproliferative diseases. Exon-8 and exon-9 are most frequently deleted, resulting in elimination at least a part of the linker and RING domains and thereby in inactivation of c-Cbl function as an E3 ligase. Several papers showed that splice site mutation (*cis*-element) of c-Cbl is responsible for exon

skipping, however, the precise mechanism of exon skipping remains unknown.

Here, I showed that c-Cbl also interacts with αPix and ubiquitinates it, but not βPix, for degradation by proteasome. Furthermore, the rat C6 and human A172 glioma cells and brain tissues of several glioma patients were found to harbor two types of exon skipping in c-Cbl: one lacking exon-9, which covers a part of the RING domain, and the other lacking both exon-9 and exson-10. Both types of the exon skipping led to inactivation of c-Cbl and stabilization and accumulation of αPix and in turn to promotion of EGF signaling and αPix-mediated cell migration and invasion. Although the precise mechanism for skipping of either enxon-9 or both exon-9 and exon-10 remains unknown, it is at least clear that the exon skipping is not due to any mutation in the corresponding splice sites as analyzed by genome sequencing of c-Cbl. Instead, the exon skipping appeared to be related with abnormal behavior of *trans*-element(s), since it could be induced under hypoxic stress conditions or when cells were grown to confluence for contact inhibition. These results

indicate that the exon skipping of c-Cbl contributes to human glioma and its malignant behavior.

MATERIALS AND METHODS

Plasmids and antibodies

The pFlag-CMV2-αPix and pFlag-CMV2-c-Cbl constructs were provided by Prof. Dong-Eun Park (Seoul National University, Seoul, Korea). They were subcloned into pcDNA-HisMax. Site-directed mutagenesis of c-Cbl was performed using mutagenesis kit (Stratagene) by following the manufacturer's instructions. c-Cbl cDNAs lacking either exon-9 or both exon-9 and exon-10 were inserted into pFlag-CMV2 or pcDNA-HisMax. Mini-gene construct harboring c-Cbl genomic DNA from exon-7 to exon-11 were inserted into pFlag-CMV2, and cDNA of GFP was fused to the 3'-end of the mini-gene. shRNA vectors for c-Cbl was purchased from Open Biosystems (# RHS4529). shαPix was cloned into pSilencer 3.1 vector (Invitrogen) using 5'-GAGGCTGGTGGGAAGGCACATTAAA-3', which is identical for αPix in all of

human, mouse, and rat. All primers were purchased from Bioneer (Daejeon, Korea).

Antibodies against Flag (Sigma), Xpress (Invitrogen), HA (Roche), c-Cbl (Santa Cruz), ERK (Cell Signaling), phospho-ERK (Cell Signaling) and tubulin (Santa Cruz) were purchased from the respective manufacturers. To generate anti-αPix antibody, I constructed a vector that can produce GST fusion of a αPix-specific region (amino acids 56-83). The fusion protein was expressed in *Escherichia coli*, purified by using GSH-Sepharose resin, and injected to rabbits. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories.

Cell culture and transfection

Cells were grown at 37°C under atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Welgene) supplemented with 1% (v/v) antibiotic-antimycotic solution (Welgene) and 10% (v/v) fetal bovine serum (Gibco).

To determine the cell density-dependent exon skipping, C6 and A172 cells were seeded at the densities of 5×10^5 cells (for low density) and 2.5×10^7 cells (for high density) onto 100 mm culture dishes. After growing the cells for 24 h, they were harvested and subjected to RT-PCR by using appropriate primers.

Primary glial cells were prepared from rat brains of post-natal day 3. Dissociated glial cells were plated in MEM containing 0.6% glucose, 1 mM pyruvate, 2 mM L-glutamine, 10% horse serum, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin.

Transfection to HEK293T and COS7 cells were carried out using JetPEI (Polyplus). Transfection to C6 and A172 cells were performed by electroporation (NEON, invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and pull-down analysis

For immunoprecipitation, cells were lysed in lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 1x protease inhibitor cocktail (Roche). Cell lysates were incubated with appropriate antibodies for 2 h at 4°C and then with 30 μl of 50% slurry of protein A-Sepharose (Sigma) for the next 1 h. The resins were collected by centrifugation, and washed three times with the lysis buffer. Bound proteins were eluted and denatured by boiling in 2x SDS-sampling buffer followed by immunoblot analysis.

For pull-down analysis, cell lysates were prepared in a lysis buffer consisting of Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.5% NP-40, and 1 x protease inhibitor cocktail. They were incubated with Ni^{2+}-NTA agarose bead (Qiagen) for 1 h at 4°C. The resins were collected by centrifugation, boiled in SDS-sampling buffer, and subjected to immunoblot analysis.

Assay for ubiquitination

HA-tagged αPix and c-Cbl, and ubiquitin were overexpressed in HEK293T cells. For detection of ubiquitinated αPix and c-Cbl, cell lysates were immunoprecipitated with anti-HA antibody under denaturing conditions, followed by immunoblot with anti-ubiquitin or anti-HA antibody.

RT-PCR and sequence analysis

Brain tissues of normal and GBM patients were obtained from NICHD brain and tissue bank at the University of Maryland, Baltimore. The identification number for each tissue is as follows: UMB # 5117 (normal), 1765 (P1), 4538 (P2), 4557 (P3), 1786 (P4), 4517 (P5), 4736 (P6), 4754 (P7), and 5371 (P8). C6 cell was provided from Dr. J.K. Roh (Seoul National University Hospital), F98 cell was provided from Dr. S.K. Kim (Seoul National University Hospital), U87 and U251 were provided from Dr. C. Choi (KAIST), A172 and Hs683 were provided from Dr. E.Y. Moon (Sejong University), and T98G was provided from Dr. H.H. Kim (Sungkyunkwan University Samsung Medical Center). Total RNAs from cell lines and brain tissues were isolated using Trizol (Invitrogen). RT-PCR was performed using SuperScript III (Invitrogen) by following the manufacturer's instructions. The resulting cDNAs were then used as templates for PCR amplification. To identify the sequences that were

skipped in c-Cbl, the following primers were used: 5'-CTTTACCCGACTCTTTCAGCCCTGGTCCTC-3' (forward) and 5'-TTGCTCCCCAGGTGGCAGTTTTGGCACAGG-3' (reverse) for both the human and rat c-Cbl. PCR products were cloned into pGEM®-T easy vector (Promega) according to the manufacturer's instructions, and all the sequencing reactions were performed by NICEM (Seoul National University).

The sequences of primers used for semi-quantitative PCR were: 5'-GAACCTATTCAGGCATGGGAAGGAGATGATATTA-3' (forward) and 5'-CTGCTGATGGTCTAAGTGGAGGTGCAGGTCGTAG-3' (reverse) for αPix, 5'- ATGGCCGGCAACGTGAAGAAGAGCTCTGGGGCCG-3' (forward) and 5'- TTCCTTTTAGTTCTGCCAGCATGTGGCTGAAGAT-3' (reverse) for c-Cbl, and 5'- ATGGATGATGATATCGCCGCGCTCGTCGTCGACA-3' (forward) and 5'- CGTAGATGGGCACAGTGTGGGTGACCCCGTCACC-3' (reverse) for β-actin.

Wound healing assay

C6 and A172 cells transfected with appropriate vectors were cultured in 5% CO_2 incubator at 37°C until they become confluent. They were scratched with an autoclaved 200 μl pipette tip, washed twice with PBS to remove debris, incubated for 24 h, and photographed using a CCD camera-equipped microscope.

Cell invasion assay

Quantitative cell invasion assay was performed using 8-μm polycarbonate inserts (Merck Millipore) in 24-well cell culture plates as described by the manufacturer. The lower chamber was filled with DMEM containing 20% FBS and 1% antibiotic-antimycotic solution and the upper chamber was filled with serum-free media. Cells (5×10^4) were added to upper chamber, and

incubated for 24 h at 37°C. Non-invaded cells were removed from the upper surface of the membrane using cotton swab. The invaded cells were fixed and stained with Differential Quik Stain kit (Polysciences). The stained cells were photographed and the invasion was assessed by counting the number of stained cells in four randomly chosen fields per sample.

Hypoxia induction

Approximately 5×10^5 C6 and A172 cells that had been transfected with mini-gene were seeded in 100 mm culture dishes. After culturing for 24 h, cells were exposed for various periods to an ambient oxygen concentration of 1%, 5% CO_2, and 94% N_2 at 37° C by using a controlled incubator monitoring CO_2/O_2 and CO_2/N_2 gas sources.

RESULTS

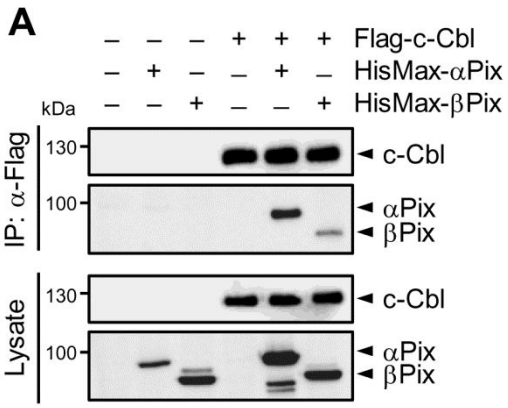
c-Cbl interacts with Pix isoforms

c-Cbl is known to interact with βPix (Flanders et al., 2003). To determine whether c-Cbl also interacts with αPix, they were overexpressed in HEK293T cells. c-Cbl bound not only to βPix but also to αPix (Figure 6A and B). It has been shown that the Arg822 and Arg829 residues in the UBA domain of c-Cbl and the Trp43 and Trp44 residues in the SH domain of βPix are involved in their interaction (Jozic et al., 2005). To determine whether the corresponding Trp residues (Trp196 and Trp197) in αPix are also required for interaction with c-Cbl, the Trp residues in αPix and the Arg residues in c-Cbl were substituted with Lys and Ala, respectively. W196K/W197K mutant αPix could not bind to c-Cbl, and R822A/R829A mutant c-Cbl could not bind to αPix (Figure 7A and B), indicating that αPix and βPix share the same conserved sites for c-Cbl binding.

Figure 6. Interaction between Pix isoforms and c-Cbl

(A) Flag-c-Cbl and HisMax-tagged Pix isoforms were expressed in HEK293T cells. After incubation for 48 h, cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-Flag and anti-Xpress antibodies.

(B) HisMax-c-Cbl and Flag-tagged Pix isoforms were expressed in HEK293T cells. After incubation for 48 h, cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-Flag and anti-Xpress antibodies.


A
– – – + + + Flag-c-Cbl
– + – – + – HisMax-αPix
– – + – – + HisMax-βPix
kDa
IP: α-Flag
130
100
c-Cbl
αPix
βPix
Lysate
130
100
c-Cbl
αPix
βPix

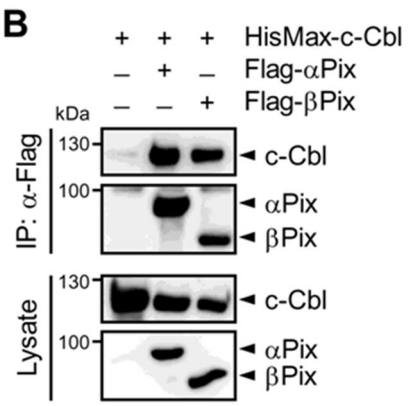
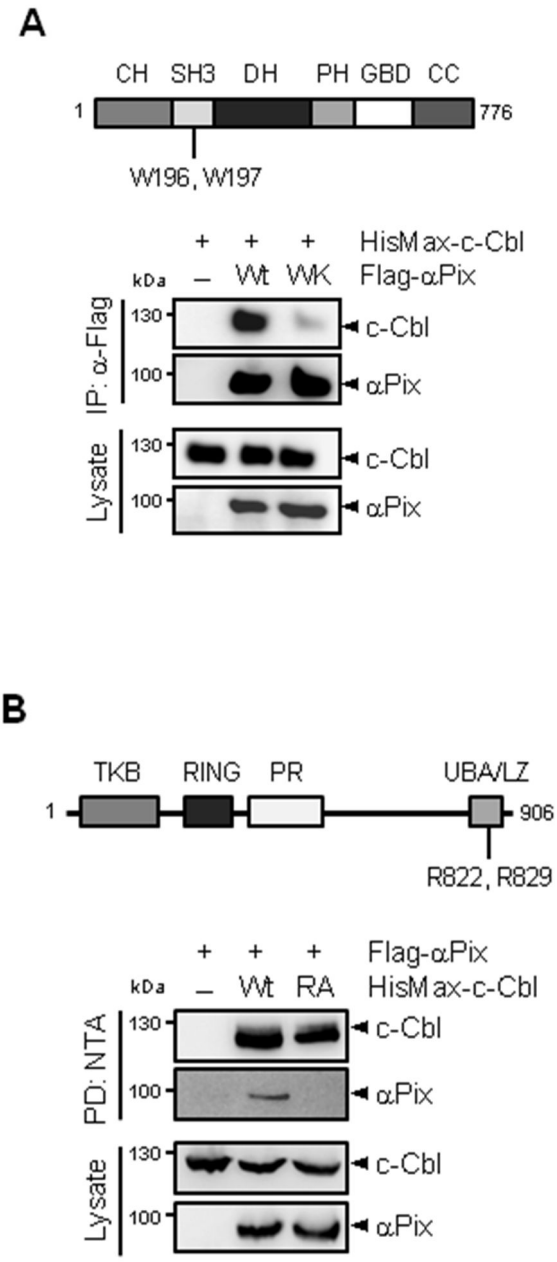

B
+ + + HisMax-c-Cbl
– + – Flag-αPix
– – + Flag-βPix
kDa
IP: α-Flag
130
100
c-Cbl
αPix
βPix
Lysate
130
100
c-Cbl
αPix
βPix

Figure 7. Identification of the binding sites in αPix and c-Cbl

(A) Putative interaction motif of αPix (196th and 197th tryptophans) were mutated to lysines (upper panel). Flag-tagged wild type αPix or mutant αPix were expressed in HEK293T cells with HisMax-c-Cbl. Cell lysates were subjected to immunoprecipitation with Flag antibody followed by immunoblot with designated antibodies (lower panel).

(B) Putative interaction motif of c-Cbl (822nd and 829th arginines) were mutated to alanines (upper panel). HisMax-tagged wild type c-Cbl or mutant c-Cbl was expressed in HEK293T cells with Flag-αPix. Cell lysates were subjected to nickel NTA pull-down followed by immunoblot with designated antibodies (lower panel).


A
CH SH3 DH PH GBD CC
1
776
W196, W197
+ + + HisMax-c-Cbl
kDa – Wt WK Flag-αPix
IP: α-Flag
130
100
c-Cbl
αPix
Lysate
130
100
c-Cbl
αPix
B
TKB RING PR UBA/LZ
1
906
R822, R829
+ + + Flag-αPix
kDa – Wt RA HisMax-c-Cbl
PD: NTA
130
100
c-Cbl
αPix
Lysate
130
100
c-Cbl
αPix

c-Cbl specifically ubiquitinates and destabilizes αPix

To determine whether c-Cbl is capable of destabilizing αPix and thereby involved in the control of cell migration, Flag-tagged αPix or βPix and HA-Ub were overexpressed in HEK293T cells with or without HisMax-c-Cbl. Despite the finding that c-Cbl could interact with both αPix and βPix, immunoprecipitation analysis revealed that ubiquitination of αPix, but not βPix, was markedly increased by c-Cbl overexpression (Figure 8). Moreover, catalytically inactive form of c-Cbl (C381A), unlike its wild-type form, could not ubiquitinate αPix (Figure 9), indicating that αPix is a specific substrate of c-Cbl.

Furthermore, the expression of a c-Cbl-specific shRNA (shc-Cbl), but not a nonspecific shRNA (shNS), abrogated αPix ubiquitination (Figure 10A). Knockdown of c-Cbl also led to a marked increase in the stability of αPix upon analysis by treatment with cycloheximide (Figure 10B). These results indicate that c-Cbl serves as a αPix-specific ubiquitin E3 ligase.

Figure 8. Ubiquitination of Pix isoforms by c-Cbl

Flag-tagged Pix isoforms were overexpressed in HEK293T cells with HisMax-c-Cbl and HA-ubiuqitin. After incubation for 48 h, 20 cells wre treated with 20μM of MG132 lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-HA and anti-Flag antibodies. Lysates were also directly probed with respective antibodies.

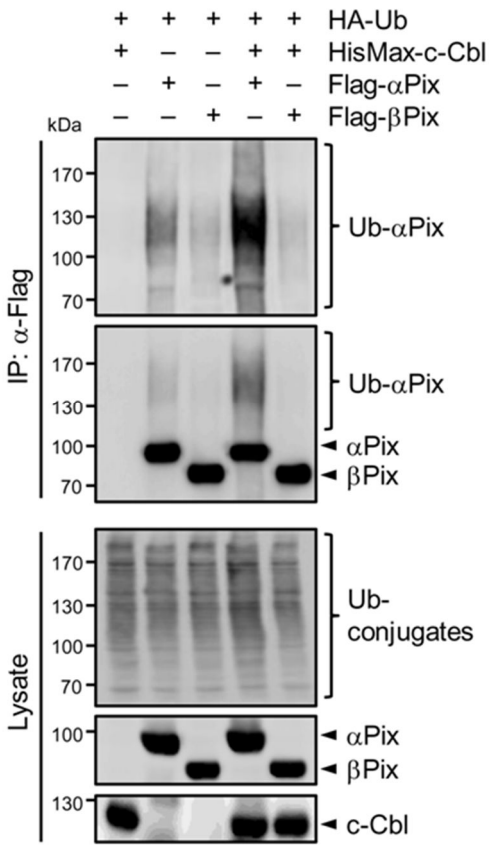

+ + + + + HA-Ub
+ – – + + HisMax-c-Cbl
– + – + – Flag-αPix
– – + – + Flag-βPix
kDa
IP: α-Flag
170
130
100
70
Ub-αPix
Ub-αPix
αPix
βPix
Lysate
Ub-
conjugates
αPix
βPix
c-Cbl

Figure 9. A c-Cbl variant, C381A, cannot ubiquitinate αPix

HisMax-tagged wild-type (Wt) and catalytically inactive form of c-Cbl (C381A; CA) were expressed in cells with HA-ubiquitin and Flag-αPix. Transfected cells were treated with 20μM of MG132. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-HA and anti-Flag antibodies. Lysates were also directly probed with respective antibodies.

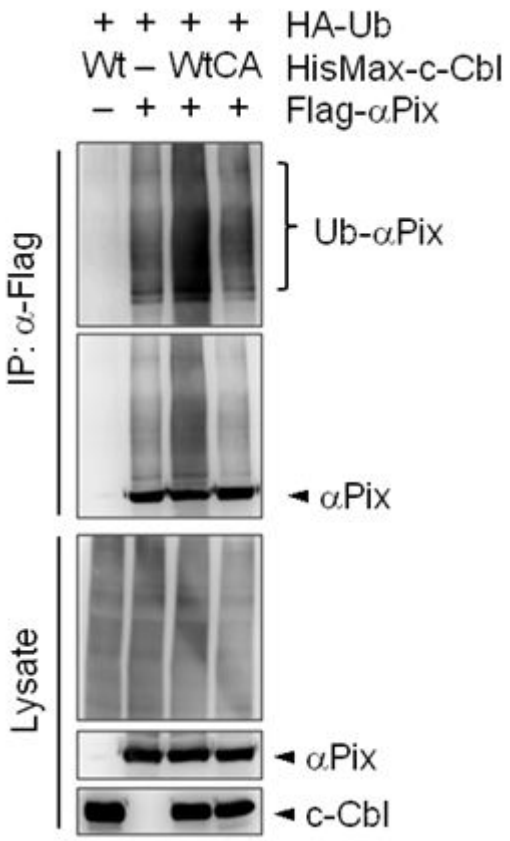
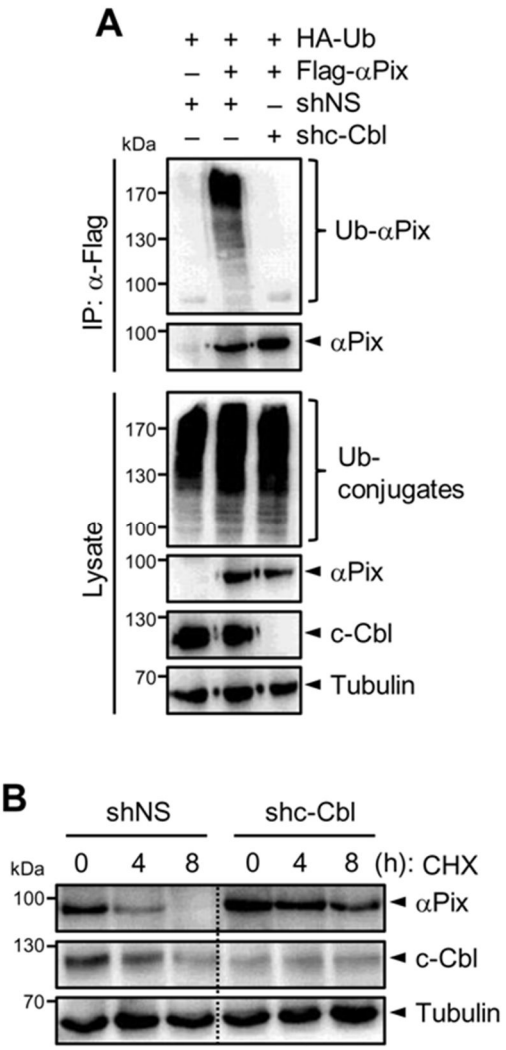

+ + + + HA-Ub
Wt – WtCA HisMax-c-Cbl
– + + + Flag-αPix
IP: α-Flag
Ub-αPix
αPix
Lysate
αPix
c-Cbl

Figure 10. Knockdown of c-Cbl stabilizes αPix.

(A) shc-Cbl or shNS was expressed in cells with Flag-αPix and HA-ubiquitin. Transfected cells were treated with 20μM of MG132. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-HA and anti-Flag antibodies. Lysates were also directly probed with respective antibodies.

(B) shc-Cbl or shNS was expressed in cells with Flag-αPix. Cells were then subjected to incubation with 200 μg/ml of cycloheximide (CHX) followed by immunoblot with anti-Flag and anti-c-Cbl antibody.


A
+ + + HA-Ub
– + + Flag-αPix
+ + – shNS
– – + shc-Cbl
kDa
IP: α-Flag
170
130
100
Ub-αPix
100
αPix
Lysate
170
130
100
Ub-
conjugates
100
αPix
130
c-Cbl
70
Tubulin
B
shNS
shc-Cbl
kDa
0 4 8 0 4 8 (h): CHX
100
αPix
130
c-Cbl
70
Tubulin

αPix is up-regulated in C6 and A172 glioma cell lines

It has been reported that both the mRNA and protein levels of αPix are up-regulated in the brain tissues of GBM patients (Yokota et al., 2006; Hua et al., 2011). To determine whether αPix expression is also up-regulated in glioma cell lines, I compared the mRNA levels of αPix in various cell lines. The expression of αPix mRNA was dramatically up-regulated in two glioma cell lines, the rat C6 and human A172, but not in other human glioma cells or HEK293T cells (Figure 11). Consistently, the expression of αPix protein could be seen only in C6 and A172 cells. I then examined whether the mRNA and protein levels of c-Cbl might also be altered in the cell lines tested. Strikingly, c-Cbl protein could not be detected only in C6 and A172 cells, despite the finding that c-Cbl mRNA was expressed to a similar extent in all cell lines tested. These results suggest that the elevation of αPix protein level in C6 and A172 cells is due to the lack of c-Cbl protein.

I next examined whether ectopic expression of c-Cbl can destabilize endogenous αPix in the rat C6 cells. αPix ubiquitination was markedly promoted by the expression of c-Cbl, but not by that of its catalytically inactive mutant (C381A), of which the active site Cys381 was replaced by Ala (Figure 12A). Furthermore, the expression of c-Cbl, but not C381A, dramatically reduced the level of αPix and this decrease could be prevented by treatment with MG132, a proteasome inhibitor (Figure 12B), indicating that the ubiquitin-proteasome system is responsible for αPix degradation. Similar results were obtained with A172 cells (Figure 13A and B). These results indicate that αPix up-regulation in C6 and A172 cells is due to the lack of its ubiquitin E3 ligase, c-Cbl.

Figure 11. Expression of αPix and c-Cbl in glioma cell lines.

The mRNA levels of αPix and c-Cbl in glioma cell lines were determined by semi-quantitative RT-PCR. Their protein levels in the cells were also determined by immunoblot with anti-αPix and anti-c-Cbl antibodies. 293 indicates HEK293T cells. C6 and F98 are rat glioma cells and the others are human glioma cells.

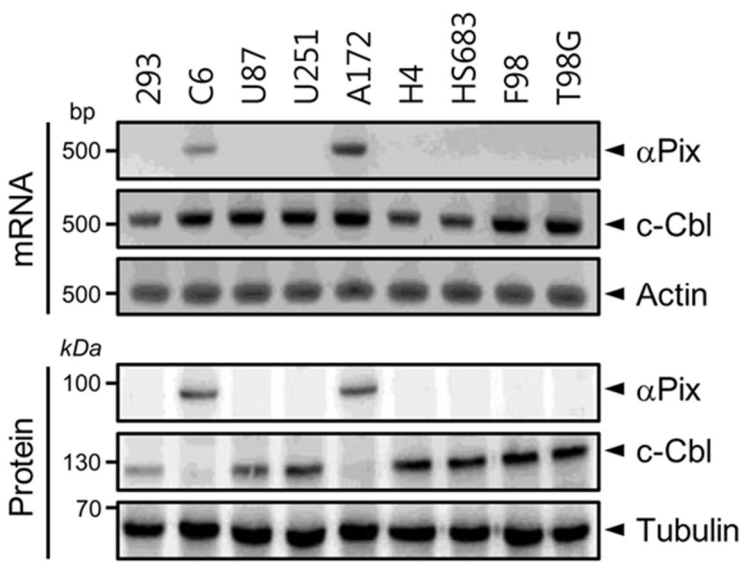

293
C6
U87
U251
A172
H4
HS683
F98
T98G
bp
mRNA
500
αPix
500
c-Cbl
500
Actin
kDa
Protein
100
αPix
130
c-Cbl
70
Tubulin

Figure 12. c-Cbl down-regulates endogenous αPix in C6 cells

(A) Flag-tagged c-Cbl (Wt) and its C381A mutant (CA) were expressed in C6 cells with HA-ubiquitin. After incubation with 20 μM MG132 for 8 h, cells were subjected to immunoprecipitation with anti-αPix antibody followed by immunoblot with anti-HA antibody.

(B) Flag-tagged c-Cbl and or its C381A mutant was expressed in C6 cells with or without 20 μM MG132. Cell lysates were then subjected to immunoblot analysis.

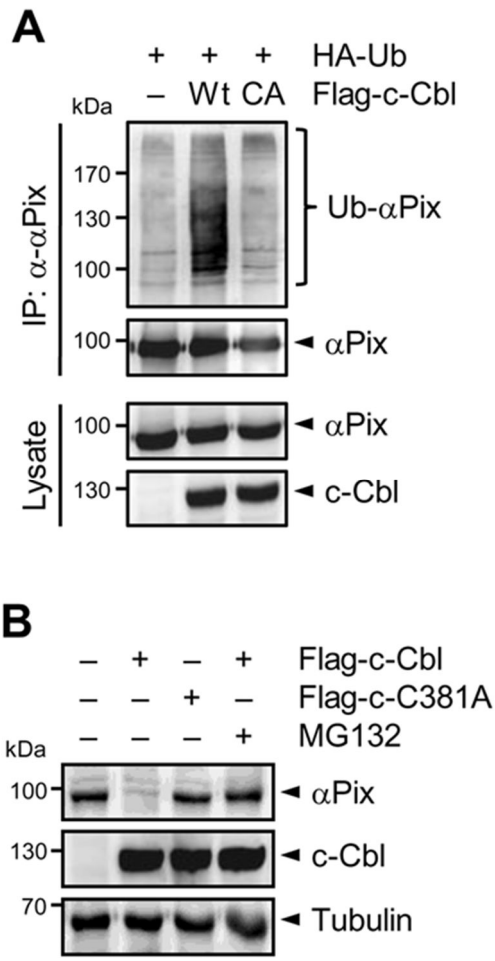
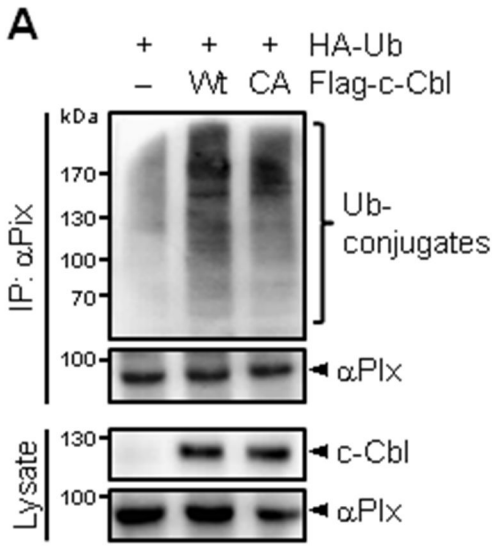
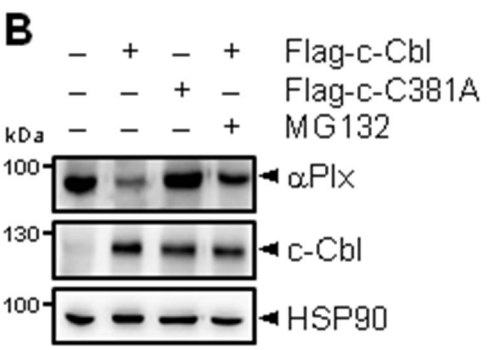

A
+ + + HA-Ub
– Wt CA Flag-c-Cbl
kDa
IP: α-αPix
170
130
100
Ub-αPix
100
αPix
Lysate
100
αPix
130
c-Cbl
B
– + – + Flag-c-Cbl
– – + – Flag-c-C381A
– – – + MG132
kDa
100
αPix
130
c-Cbl
70
Tubulin

Figure 13. c-Cbl down-regulates endogenous αPix in A172 cells

The experiments were performed as in Figure 12 but using A172 cells.


A
+ + + HA-Ub
– Wt CA Flag-c-Cbl
kDa
170
130
100
70
IP: αPix
Ub-
conjugates
100
αPIx
Lysate
130
c-Cbl
100
αPIx


B
– + – + Flag-c-Cbl
– – + – Flag-c-C381A
kDa – – – + MG132
100
αPIx
130
c-Cbl
100
HSP90

Exon skipping of c-Cbl in C6 and A172 cell lines and brain tissues of several GBM patients

Since both C6 and A172 cells express c-Cbl mRNA but not c-Cbl protein, it appeared possible that c-Cbl in the glioma cells might be mutated and destabilized, resulting in αPix accumulation. To test this possibility, total mRNAs were prepared from the cells, and subjected to RT-PCR followed by cDNA sequencing. Comparison with the sequence of c-Cbl cDNA from database revealed that cDNAs from the glioma cells lack either one or two specific exon sequences (Figure 14A): type I lacking exon-9 and type II lacking both exon-9 and exon-10 (Figure 14B). To show more clearly the exon skipping, PCR was performed using primers directed to the sequences in the region between exon-4/5 and exon-12. Both types of the exon skipping could evidently be seen in the cDNAs from the glioma cells (Figure 15A). These results demonstrate that both rat C6 and human A172 glioma cells harbor two

types of exon skipped forms in c-Cbl.

I next examined whether the same or similar exon skipping could be detected in brain tissues of GBM patients, which were obtained from NICHD Brain and Tissue Bank (the University of Maryland, Baltimore). Total mRNAs were prepared from the tissues, and subjected to RT-PCR followed by cDNA sequencing. As was found in C6 and A172 cells, the expression of αPix mRNA was dramatically up-regulated in brain tissues from three GBM patients (P1-P3), although not in five other GBM tissues or in a normal brain tissue (Figure 15B). In the same tissues, αPix protein was strongly expressed, whereas c-Cbl protein could not be detected. Furthermore, P1-P3 patients were found to have the same type I and type II exon skipping in their cDNAs, as were found in C6 and A172 cells. PCR analysis using the same primers used in Figure 7C confirmed the exon skipping in c-Cbl cDNAs from P1-P3 patients (Figure 15C). These findings suggest that c-Cbl exon skipping contributes to human GBM. The accession numbers for the cDNAs of c-Cbl

mutants are: KJ944831 (type 1) and KJ944832 (type II) from A172, KJ944833 (type I) and KJ944834 (type II) from C6, KJ944835 (type I) and KJ944836 (type II) from P1, KJ944837 (type I) and KJ944838 (type II) from P2, and KJ944839 (type I) and KJ944840 (type II) from P3.

Figure 14. Schematic diagram of c-Cbl exon skipping

(A) c-Cbl cDNAs of C6 cells were subjected to sequence analysis. The primary sequences of wild-type (Wt) and exon skipped c-Cbl (type I and II) are shown with their functional domains. TKB and PR indicate tyrosine kinase-binding domain and Pro-rich domain, respectively.

(B) Schematic diagram indicates two types of c-Cbl exon skipping.

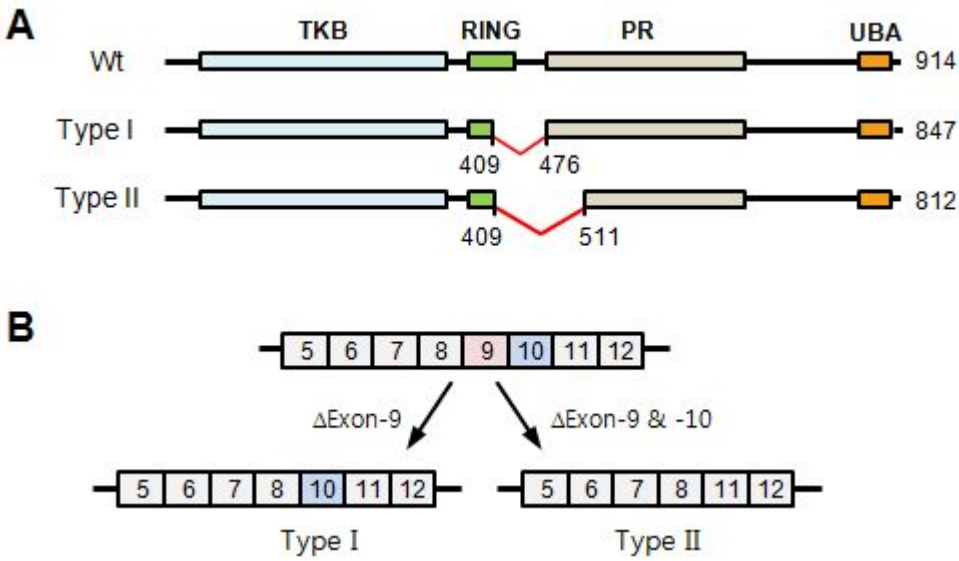
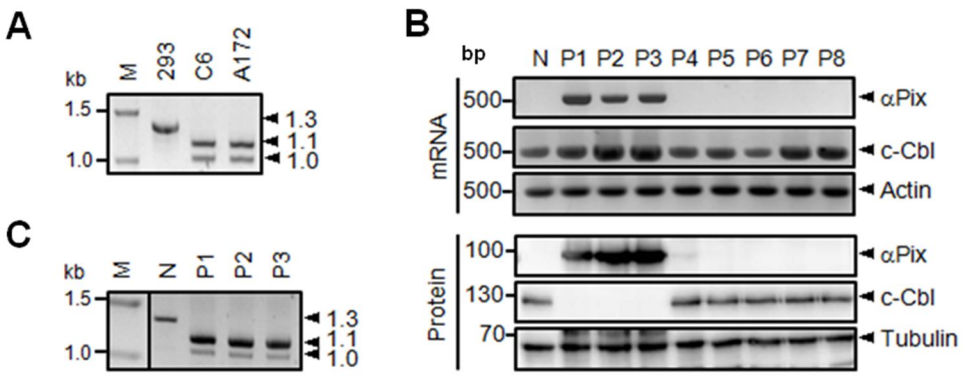

A
TKB
RING
PR
UBA
Wt
914
Type I
847
409
476
Type II
812
409
511
B
5
6
7
8
9
10
11
12
ΔExon-9
ΔExon-9 & -10
Type I
Type II

Figure 15. c-Cbl exon skipping in glioma cells and brain tissues of GBM patients

(A and C) Using cDNAs as templates, PCR was performed with primers directed to a region between exon-4/5 and exon-12. The length of RCR product from wild-type c-Cbl cDNA (i.e., from HEK293T cells) was about 1.3 kb. Skipping of exon-9 and both exon-9 and exon-10 led to the generation of PCR products with approximate sizes of 1.1 and 1.0 kb, respectively.

(B) Total mRNAs were isolated from brain tissues from normal (N) and GBM patients (P1-P8) by using Trizol. They were then subjected to RT-PCR using primers specific to c-Cbl and αPix (upper panel). The same brain tissues were homogenized in a lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and 1x protease inhibitor cocktail. After centrifugation, soluble fractions were subjected to immunoblot analysis (lower panel).


A
kb
M
293
C6
A172
1.5
1.0
1.3
1.1
1.0
C
kb
M
N
P1
P2
P3
1.5
1.0
1.3
1.1
1.0
B
bp
N P1 P2 P3 P4 P5 P6 P7 P8
mRNA
500
αPix
500
c-Cbl
500
Actin
Protein
100
αPix
130
c-Cbl
70
Tubulin

c-Cbl proteins generated by exon skipping are inactivated and destabilized.

Skipping of c-Cbl exon-9 found in both types resulted in elimination of approximately one-half of the RING domain (see Figure 14A), which is required for the catalytic function of c-Cbl as an ubiquitin E3 ligase. Thus, it is likely that c-Cbl generated by both types of exon skipping might be unable to ubiquitinate αPix. To confirm this, type I and type II cDNAs (referred to as Δ1 and Δ2, respectively) were cloned into pFlag-CMV2 vector. Flag-tagged c-Cbl, Δ1, and Δ2 were then overexpressed in HEK293T cells with αPix and ubiquitin. Immunoprecipitation analysis showed that αPix was ubiquitinated by c-Cbl, but not by Δ1 and Δ2 (Figure 16), indicating that the exon skipping leads to inactivation of c-Cbl.

Notably, no c-Cbl protein could be detected in C6 and A172 cells and in brain tissues of P1-P3 patients, despite the finding that c-Cbl mRNAs are

normally expressed (see Figure 11 and 15B). However, the endogenous protein expression level of c-Cbl in C6 and A172 was slightly increased upon MG132 treatment (data not shown). These findings raise a possibility that Δ1 and Δ2 are unstable due to the exon skipping. To test this possibility, experiments were performed as in Figure 16, but using C6 cells and without αPix expression. Unlike wild-type c-Cbl, both Δ1 and Δ2 were heavily ubiquitinated (Figure 17). Similar results were obtained when Δ1 and Δ2 were expressed in A172 cells (Figure 18). Moreover, the stability of Δ1 and Δ2 was dramatically reduced as compared to that of wild-type c-Cbl (Figure 19A and B). These results indicate that the lack of c-Cbl protein in C6 and A172 cells and brain tissue of P1-P3 patients is due to the exon skipping, which destabilize the E3 ligase.

Figure 16. c-Cbl generated by exon skipping in C6 is inactivated.

Flag-tagged c-Cbl (Wt), Δ1, and Δ2 were expressed in HEK293T cells with HisMax-αPix and HA-ubiquitin. After incubation with 20 μM MG132 for 8 h, cell lysates were subjected to pull-down with NTA resins followed by immunoblot with anti-HA and anti-Xpress antibodies.

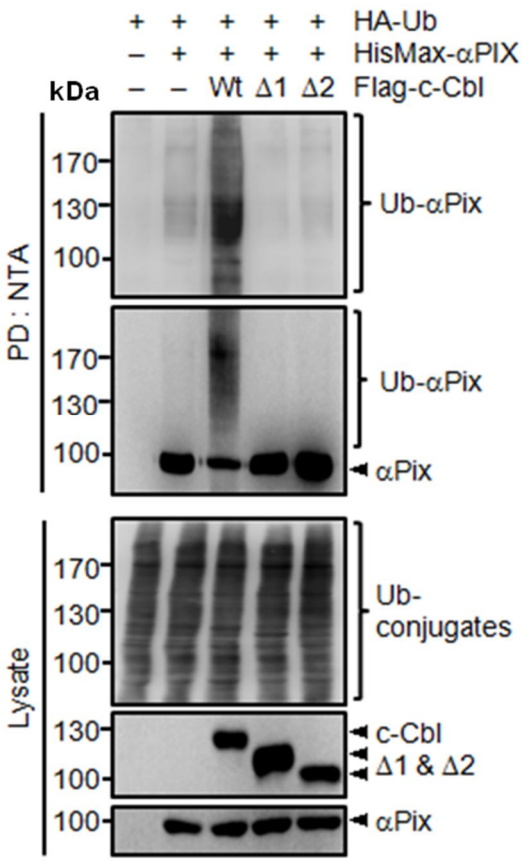

+ + + + + HA-Ub
– + + + + HisMax-αPIX
kDa – – Wt Δ1 Δ2 Flag-c-Cbl
PD : NTA
170
130
100
Ub-αPix
170
130
100
Ub-αPix
αPix
Lysate
170
130
100
Ub-
conjugates
130
100
c-Cbl
Δ1 & Δ2
100
αPix

Figure 17. Exon skipping leads to ubiquitination of c-Cbl in C6 cells

Flag-tagged c-Cbl, Δ1, and Δ2 were expressed in C6 cells with HA-ubiquitin. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblot with anti-HA and anti-Flag antibodies. Lysates were also directly probed with respective antibodies.

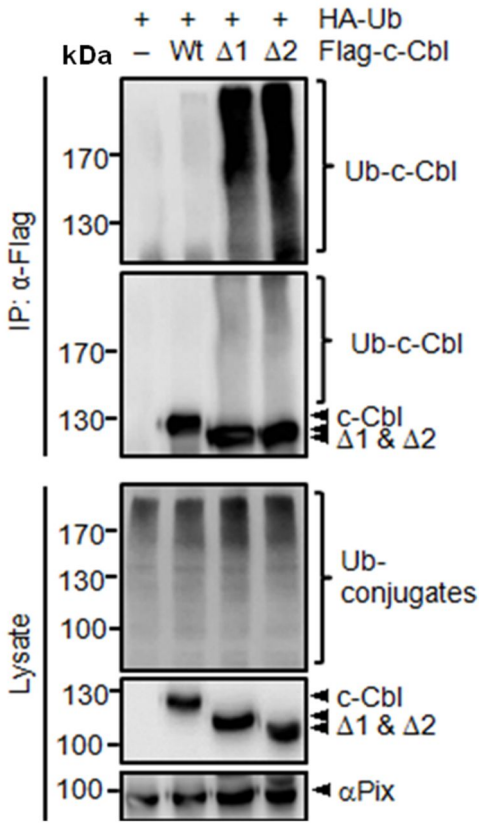

+ + + + HA-Ub
kDa – Wt Δ1 Δ2 Flag-c-Cbl
IP: α-Flag
170
130
Ub-c-Cbl
170
130
Ub-c-Cbl
c-Cbl
Δ1 & Δ2
Lysate
170
130
100
Ub-
conjugates
130
100
c-Cbl
Δ1 & Δ2
100
αPix

Figure 18. Exon skipping leads to ubiquitination of c-Cbl in A172 cells

The same experiment was performed as figure 17 by using A172 cells

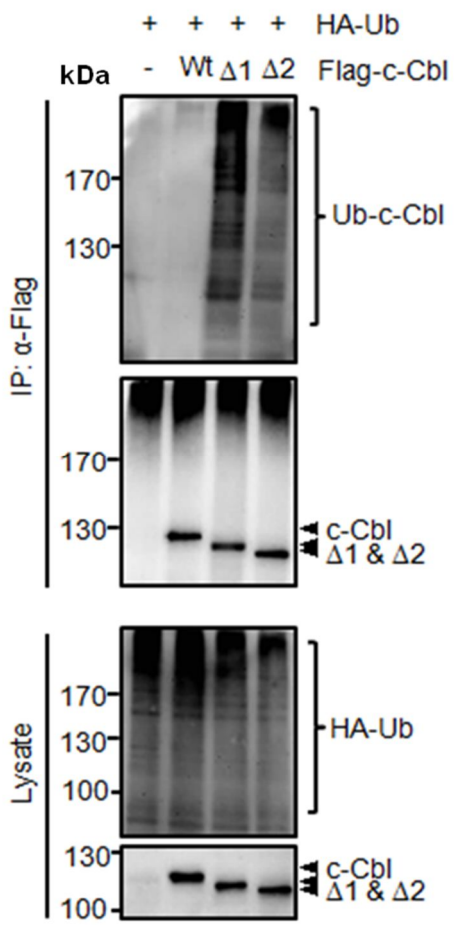
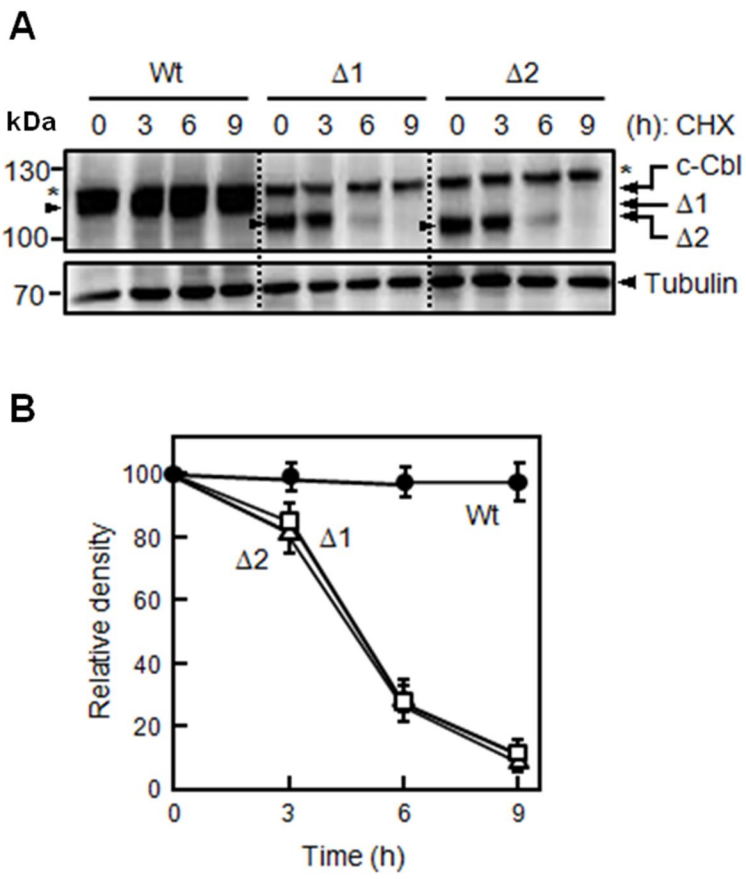

+ + + + HA-Ub
kDa - Wt Δ1 Δ2 Flag-c-Cbl
IP: α-Flag
170
130
Ub-c-Cbl
170
130
c-Cbl
Δ1 & Δ2
Lysate
170
130
100
HA-Ub
130
100
c-Cbl
Δ1 & Δ2

Figure 19. Exon skipping leads to destabilization of c-Cbl

(A) C6 cells expressing Flag-tagged c-Cbl, Δ1, and Δ2 were incubated with 200 μg/ml of cycloheximide. Cell lysates were then subjected to immunoblot with anti-Flag antibody. Asterisk (*) indicates nonspecific band.

(B) The c-Cbl bands in (A) were scanned using a densitometer and their densities were quantified by using “Image J” software. The density seen at each ‘0’ time point was expressed as 100% and the others as its relative values. Data are the mean ± s.d. (n = 3).


A
Wt
Δ1
Δ2
kDa
0 3 6 9 0 3 6 9 0 3 6 9
(h): CHX
130
100
70
*
c-Cbl
Δ1
Δ2
Tubulin
B
Relative density
100
80
60
40
20
0
0
3
6
9
Time (h)
Wt
Δ1
Δ2

c-Cbl exon skipping up-regulates EGF signal

c-Cbl is known to play an important role in switching off EGF signal by down-regulating EGFR (Niemeyer et al., 2010). Therefore, I examined whether the c-Cbl exon skipping leads to sustained activation of EGFR. EGF treatment resulted in persistent phosphorylation of ERK even after 2 h in C6 cells, unlike in glial cells, of which ERK activation transiently occurred only at 5 min after the treatment (Figure 20A and B). Furthermore, ERK phosphorylation was significantly reduced when C6 cells were complemented with wild-type c-Cbl (Figure 20C and D). In addition, overexpression of ∆1 and ∆2 in COS7 cells led to an increase in ERK phosphorylation (Figure 21A and B), suggesting that the overexpressed ∆1 and ∆2 could act dominant negatively to endogenous c-Cbl in down-regulation of EGF signaling.

Figure 20. c-Cbl exon skipping leads to persistent EGFR activation in C6 cells

(A) C6 and glial cells treated with 0.5 ng/ml of EGF were incubated for various periods, and subjected to immunoblot with indicated antibodies.

(B) The phospho-ERK bands in (A) were scanned and quantified. The density seen at each '0' time point was expressed as 100% and the others as its relative values.

(C) Experiments were performed as in (A), except the use of C6 cells transfected with an empty vector (control) or a vector expressing Flag-c-Cbl.

(D) The phospho-ERK bands in (C) were scanned and quantified. Data of (B) and (D) are the mean ± s.d. (n = 3).

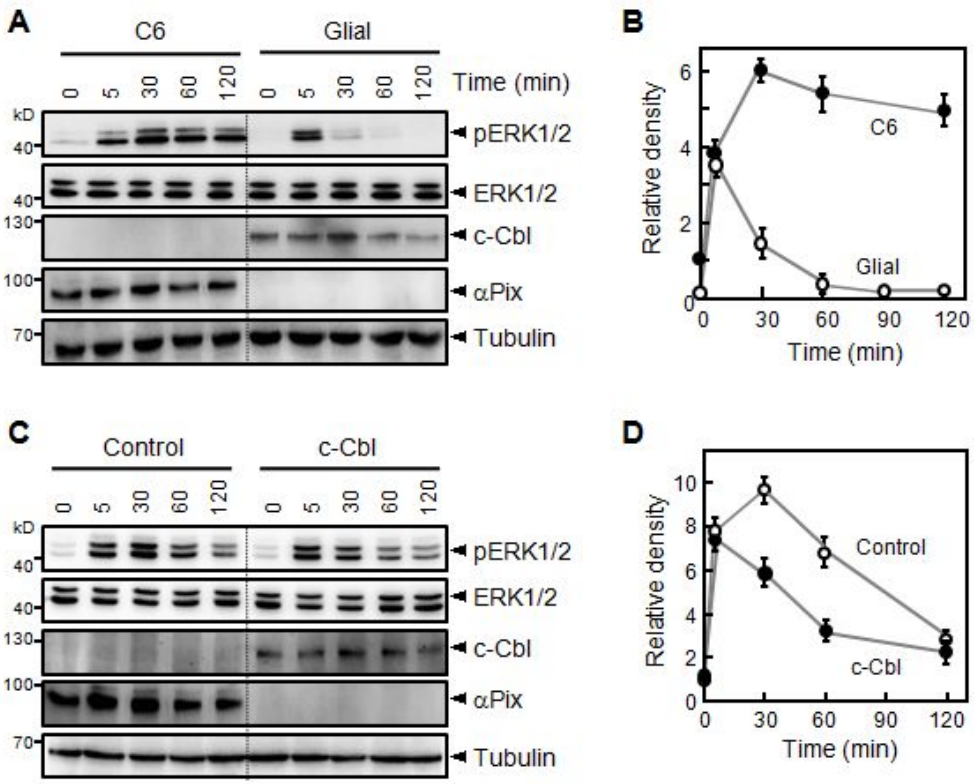
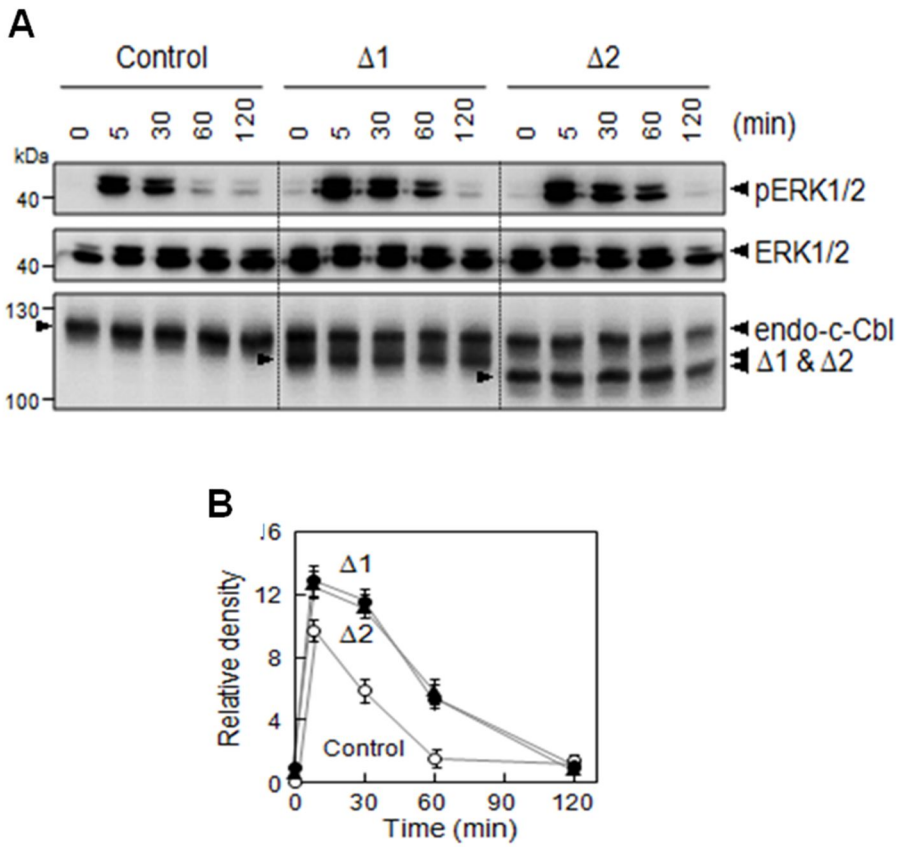

A
C6
Glial
Time (min)
0
5
30
60
120
kD
40
40
130
100
70
pERK1/2
ERK1/2
c-Cbl
αPix
Tubulin
B
Relative density
C6
Glial
Time (min)
C
Control
c-Cbl
D
Control
c-Cbl

Figure 21. Δ1 and Δ2 generated by exon skipping act dominant negatively to endogenous c-Cbl in down-regulation of EGF signaling.

HisMax-tagged Δ1 and Δ2 were expressed in COS7 cells. After incubation of 24 h, 0.5 ng/ml of EGF were treated for the indicated periods. Cell lysates were then subjected to immunoblot analysis.


A
Control
Δ1
Δ2
0
5
30
60
120
(min)
kDa
40
pERK1/2
ERK1/2
130
100
endo-c-Cbl
Δ1 & Δ2
B
Relative density
Δ1
Δ2
Control
0
4
8
12
16
0
30
60
90
120
Time (min)

c-Cbl exon skipping promotes αPix-mediated cell migration and invasion

αPix is known to promote cell migration (Hua et al., 2011), a critical process required for malignant behavior of cancer cells, such as glioma and AGS gastric cancer cells. To determine whether the up-regulated αPix in C6 cells (see Figures 11 and 15B) could indeed promote the cells' ability to migrate, wound-healing assay was performed. Expression of a αPix-specific shRNA (shαPix) markedly reduced the ability of C6 cells in migration as compared to that of shNS (Figure 22). Moreover, overexpression of c-Cbl, but not its inactive variant (C381A), reduced the cell migration concurrent with a decrease in αPix level (Figure 23), indicating that αPix is at least in part responsible for C6 cell migration.

I next examined whether αPix-mediated cell migration promotes the ability of C6 cells in invasion. Matri-gel analysis revealed that the invasive activity of

C6 cells was markedly reduced by shαPix expression, but could be restored by co-expression of shRNA-insensitive αPix (Figure 24). Moreover, overexpression of c-Cbl, but not the C381A mutant, strongly inhibited the invasive activity. Similar results were obtained when A172 cells were used for cell migration and invasion assays (Figure 25). Collectively, these results strongly suggest that the malignant behavior of the glioma cells is mediated by up-regulated expression of αPix due to c-Cbl exon skipping.

Figure 22. Knockdown of αPix down-regulates the migration of C6 cells.

(A) Cells expressing shNS or shαPix were subjected to wound healing assay.

(B) Empty areas in (A) were quantified by using “Image J” software. The area seen at ‘0’ time with cells transfected with shNS was expressed as 100% and the others as its relative values.

(C) Cells used in (A) were subjected to immunoblot analysis to verify αPix knockdown.

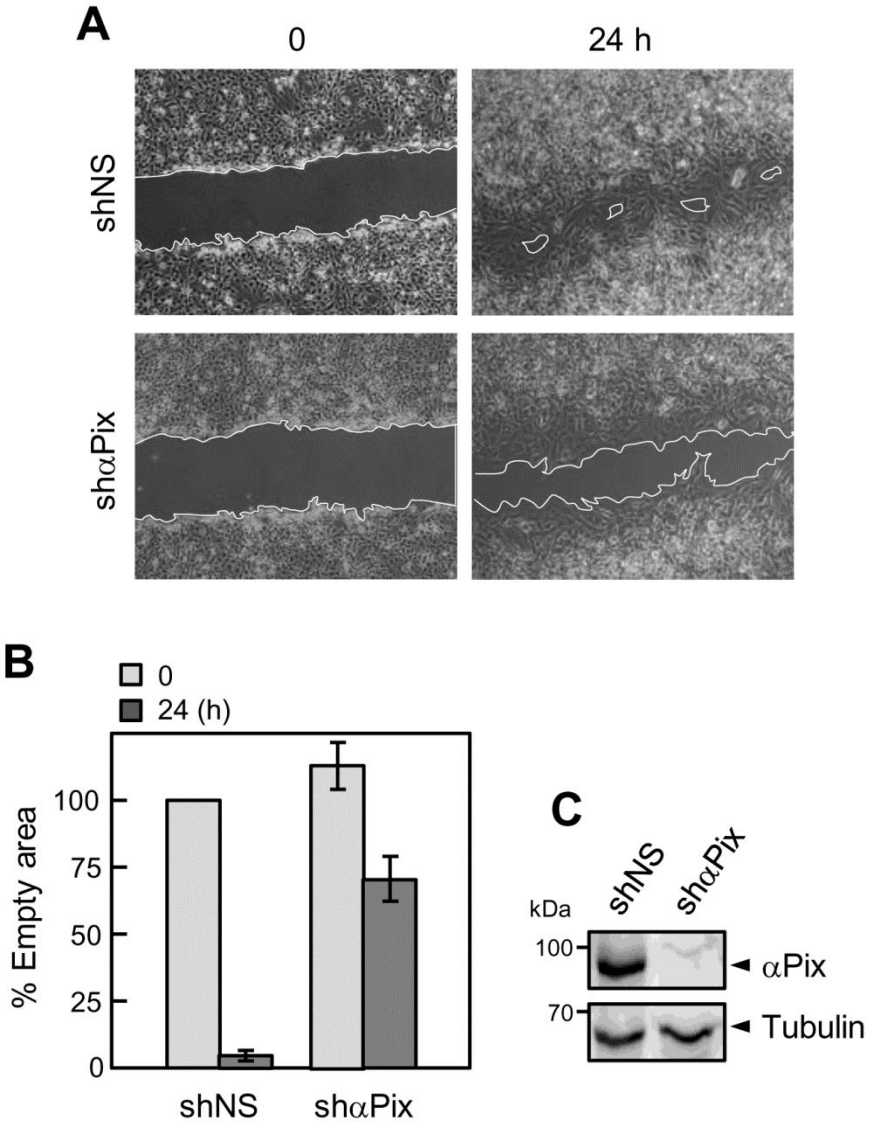

A
0
24 h
shNS
shαPix
B
0
24 (h)
% Empty area
100
75
50
25
0
shNS
shαPix
C
shNS
shαPix
kDa
100
αPix
70
Tubulin

Figure 23. Overexpression of c-Cbl down-regulates the migration of C6 cells

(A) Cells expressing c-Cbl (Wt) or its C381A mutant (CA) were subjected to wound healing assay. Mock indicates the cells transfected with an empty vector.

(B) Empty areas were quantified as in (B), but by setting the empty area of mock cells at '0' time as 100%.

(C) Cells used in (A) were subjected to immunoblot analysis to verify the expression of αPix and c-Cbl proteins. Data are the mean ± s.d. (n = 3).

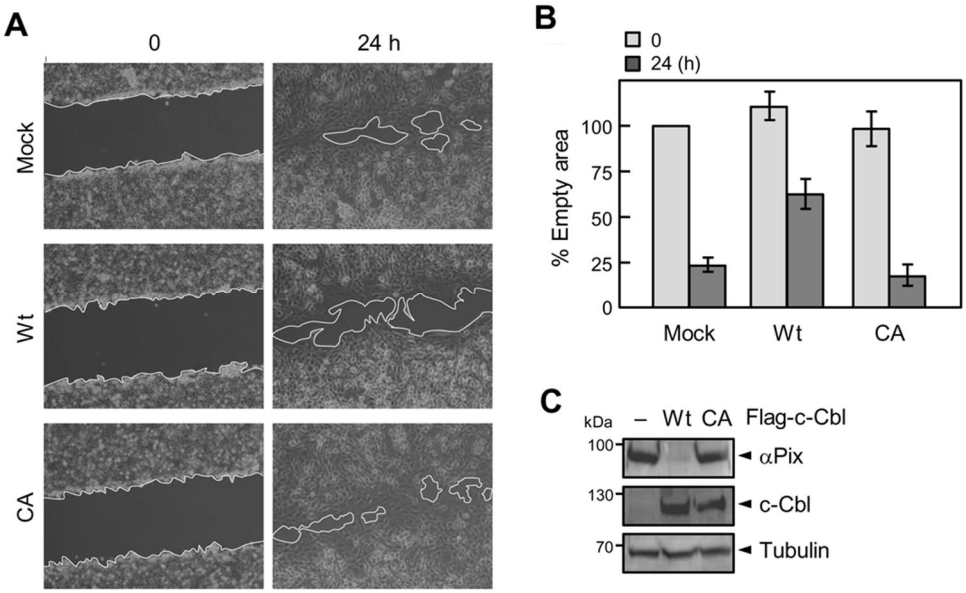

A
0
24 h
Mock
Wt
CA
B
0
24 (h)
% Empty area
100
75
50
25
0
Mock
Wt
CA
C
kDa
–
Wt
CA
Flag-c-Cbl
100
αPix
130
c-Cbl
70
Tubulin

Figure 24. c-Cbl exon skipping promotes the ability of C6 cells in invasion.

(A) Cells expressing shNS or shαPix (upper panel) and Flag-tagged c-Cbl or its C381A mutant (CA) (lower panel) were subjected to cell invasion assay. αPix* indicates shRNA-insensitive αPix.

(B) From the data of (A), the number of cells in four independently chosen fields were counted and averaged.

(C) Cells used in (A) were subjected to immunoblot analysis to verify the expression of proteins.

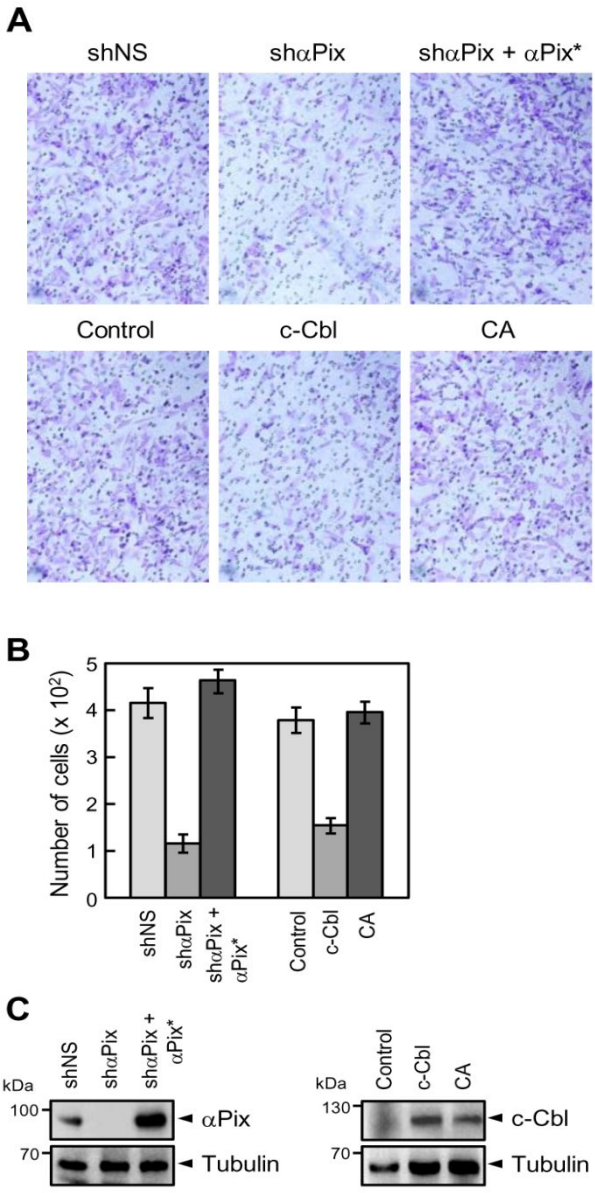
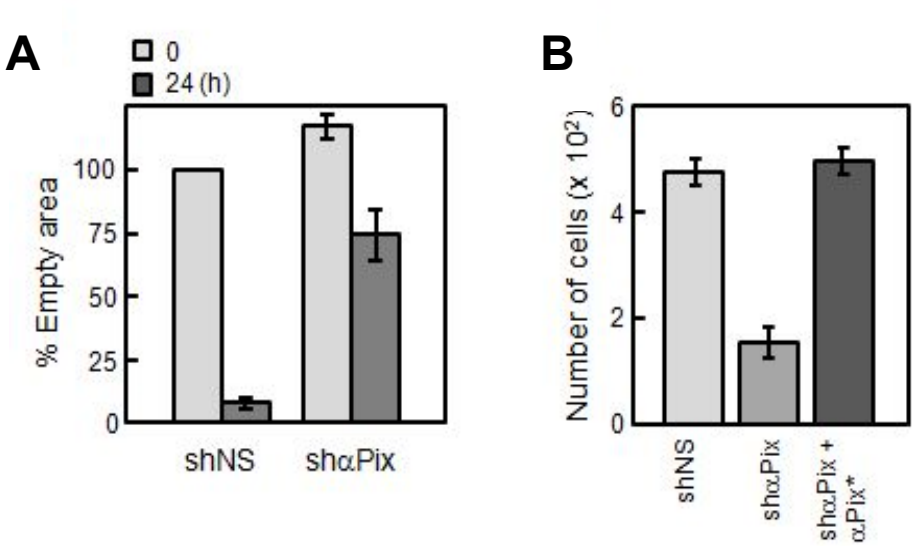

A
shNS
shαPix
shαPix + αPix*
Control
c-Cbl
CA
B
Number of cells (x 10²)
0
1
2
3
4
5
shNS
shαPix
shαPix + αPix*
Control
c-Cbl
CA
C
shNS
shαPix
shαPix + αPix*
kDa
100
70
αPix
Tubulin
Control
c-Cbl
CA
kDa
130
70
c-Cbl
Tubulin

Figure 25. Knockdown of αPix down-regulates the migration and invasion of A172 cells.

(A) Cells expressing shNS or shαPix were subjected to wound healing assay as in the Figure 22. The empty area seen at '0' time with cells transfected with shNS was expressed as 100% and the others as its relative values. Data are the mean ± s.d. (n = 3).

(B) Cells expressing shNS or shαPix with and without shRNA-insensitive αPix were subjected to cell invasion assay as in Figure 24. The number of cells in four independently chosen fields were counted and averaged.


A
0
24 (h)
% Empty area
100
75
50
25
0
shNS
shαPix
B
Number of cells (x 10²)
6
4
2
0
shNS
shαPix
shαPix +
αPix*

Confluency and hypoxia cause exon skipping of c-Cbl

Exon skipping is commonly generated by mutation(s) in splice site (*cis*-element) or sometimes by defect(s) of splicing machinery (*trans*-element). To examine the mechanism for c-Cbl exon skipping, I selected one patient tissue, which harbors exon skipping in cDNA of c-Cbl, and carried out sequence analysis of its genomic DNA covering the region between exon-7 and exon-11 (including introns). However, no putative splice site mutation was found in the region, suggesting that c-Cbl exon skipping is due to certain unknown defect(s) in *trans*-element.

To confirm this possibility, I cloned the region between exon-7 and exon-11 of c-Cbl genomic DNA (including the introns) into pFlag-CMV2 and fused GFP to the 3'-end of exon-11 (Figure 26A) as described in Materials and methods. The cloned mini-genes were then transfected into various glioma cell lines,

and subjected to RT-PCR by using primers specific to exon-7 and GFP. Consistent with the finding that only C6 and A172 cells lack c-Cbl, exon skipping of the exogenously supplied mini-gene could be observed only in C6 and A172 cells (Figure 26B). These results indicate that unknown defects of *trans*-element in C6 and A172 cells are responsible for the exon skipping.

In an attempt to determine environmental factors that could induce c-Cbl exon skipping, C6 and A172 cells transfected with the mini-gene were grown to confluent state and exposed to hypoxic stress, both of which are typical environment of cancer cells. As shown in Figure 27A, the increase in cell density to confluence led to an increase in c-Cbl exon skipping. Exposure to hypoxic stress also induced the exon skipping (Figure 27B). These results suggest that confluency and hypoxia somehow influence certain *trans*-element to trigger c-Cbl exon skipping. These results also suggest that glioma cells overcome confluency and hypoxia by exon skipping of c-Cbl for their survival and malignant behavior.

Figure 26. Exon skipping of the exogenously supplied mini-gene could be observed only in C6 and A172 cells.

(A) Schematic diagram of mini-gene containing the region between exon-7 and exon-11 of c-Cbl genomic DNA (including the introns).

(B) The mini-gene was transfected into HEK293T cells and various glioma cell lines. After incubation of the cells for 2 d, they were grown to high density (i.e., until > 70% area of culture dish were occupied by cells), and treated with Trizol. Total RNAs were then subjected to RT-PCR by using the primers specific to c-Cbl exon-7 (forward) and to GFP (reverse). Note that different primers were used from Figure 15.

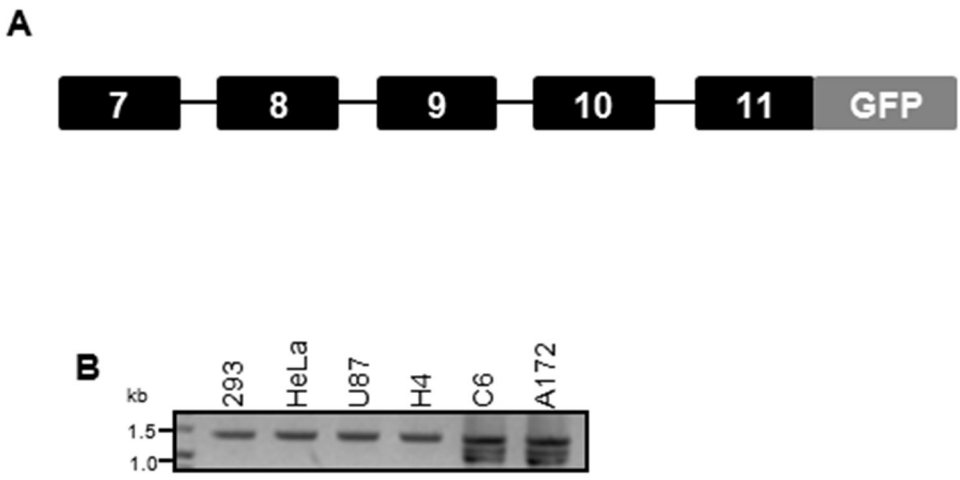
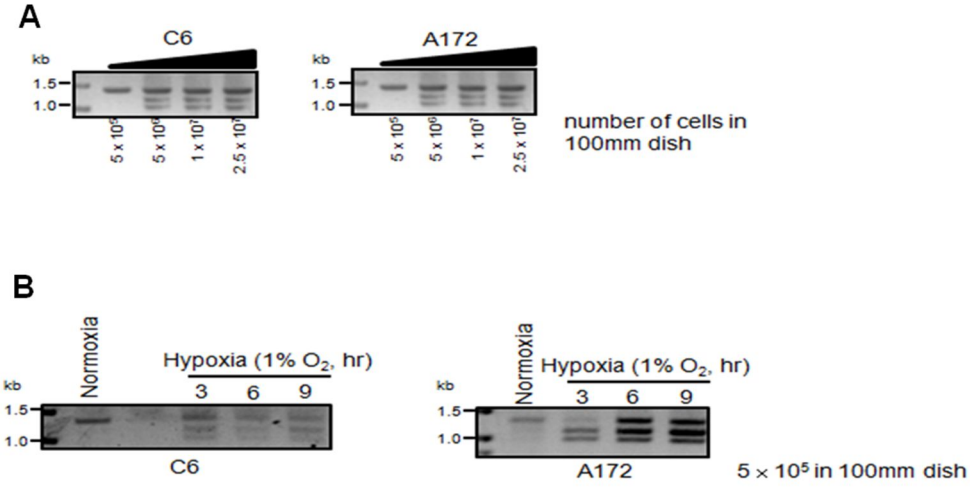

A
7
8
9
10
11
GFP
B
kb
293
HeLa
U87
H4
C6
A172
1.5
1.0

Figure 27. Confluency and hypoxia induce exon skipping of c-Cbl.

(A) C6 and A172 cells transfected with the mini-gene were seeded in 100 mm culture dishes at the indicated densities. After incubation for 24 h, RT-PCR was performed as in Figure 26.

(B) C6 and A172 cells were prepared as in (A). They were seeded in 100 mm culture dishes as the indicated cell density. After incubation for 24 h, cells were incubated under hypoxic condition as described Materials and methods, followed by RT-PCR as in Figure 26.


A
C6
A172
kb
1.5
1.0
5 x 10⁵
5 x 10⁶
1 x 10⁷
2.5 x 10⁷
number of cells in
100mm dish
B
Normoxia
Hypoxia (1% O2, hr)
3
6
9
C6
A172
5 × 10⁵ in 100mm dish

DISCUSSION

In the present study, I demonstrated that c-Cbl serves as an ubiquitin E3 ligase for degradation of αPix. Intriguingly, C6 and A172 glioma cells were found to be unable to express c-Cbl, which leads to a marked accumulation of αPix. The accumulated αPix promoted cell migration and invasion, which would provoke the malignant behavior of the cancer cells. As c-Cbl is known to down-regulate EGFR (Wong et al., 2002), the lack of c-Cbl in C6 and A172 cells would lead to sustained activation of the receptor tyrosine kinase, which would in turn promote cell proliferation and tumorigenesis. Collectively, these findings demonstrate that c-Cbl plays a crucial role in down-regulation of αPix and the lack of c-Cbl in C6 and A172 cells is responsible for their malignant behavior.

Of note, however, is the finding that αPix mRNA was not expressed in the

glioma cells tested in this study except C6 and A172, whereas both c-Cbl mRNA and c-Cbl protein were normally expressed. Thus, it is unlikely that the absence of αPix protein in those cells is due to c-Cbl-mediated destabilization. Interestingly, tissue transglutaminase (tTG) has been shown to up-regulate EGF signaling by preventing c-Cbl-mediated EGFR ubiquitination, and tTG is up-regulated in several glioma cells including U87 and T98G (Zhang et al., 2013). However, U87, U251, F98, and T98G cells lacking αPix are known to be highly invasive, although H4 and HS683 cells are derived from low-grade gliomas (Ohgaki and Kleihues, 2005; Liu et al., 2011). Thus, αPix-independent mechanism(s) appears to also operate in the malignant glioma cell lines that lack of αPix.

Human myeloid neoplasms have been shown to be associated with a variety of Cbl mutations, including missense mutations, frame-shift mutations, insertions, deletion mutations (mostly leading to elimination of a part or entire portion of exon-8), and primary transcript splicing mutations. Recently, it has

been shown that Cbl mutations also contribute to the pathogenesis of solid tumors (Tan et al., 2010). Somatic mutations were found in 8 of non-small cell lung tumors out of 119 patients, although only one was inside the linker and RING-finger regions unlike the mutations that contribute to myeloid neoplasm are mostly within the regions. Thus, glioblastoma appear to represent the second example of solid tumor associated with Cbl mutations. In this respect, it would be of interest to see if disruption of Cbl function may also contribute to the pathogenesis of other solid tumors.

Of particular interest in this study is the finding that two types of c-Cbl exon skipping occur in C6 and A172 glioma cells and brain tissues of several GBM patients: type I lacking exon-9 and type II lacking both exon-9 and exon-10. These exon skipping abrogated the ubiquitin E3 ligase function of c-Cbl, resulting in stabilization of αPix. The accumulated αPix promoted cell migration and invasion (Figures 10 and 11), both of which are typical malignant behavior of cancer cells and tissues. Thus, it appears that the exon

skipping of c-Cbl critically contribute to human glioma and its malignant behavior.

Both types of c-Cbl exon skipping were found to generate much more unstable products than its wild-type form. However, it is unlikely that the catalytic activity of c-Cbl is responsible for ubiquitination and destabilization of itself, because both products lack most part of the RING domain. It has been reported that c-Cbl can be ubiquitinated by the HECT-type ubiquitin E3 ligases, such as Itch or Nedd4, for proteasomal degradation (Magnifico et al., 2003). Thus, it appears possible that the ligases preferentially ubiquitinate the c-Cbl generated by exon skipping over its wild-type E3 ligase.

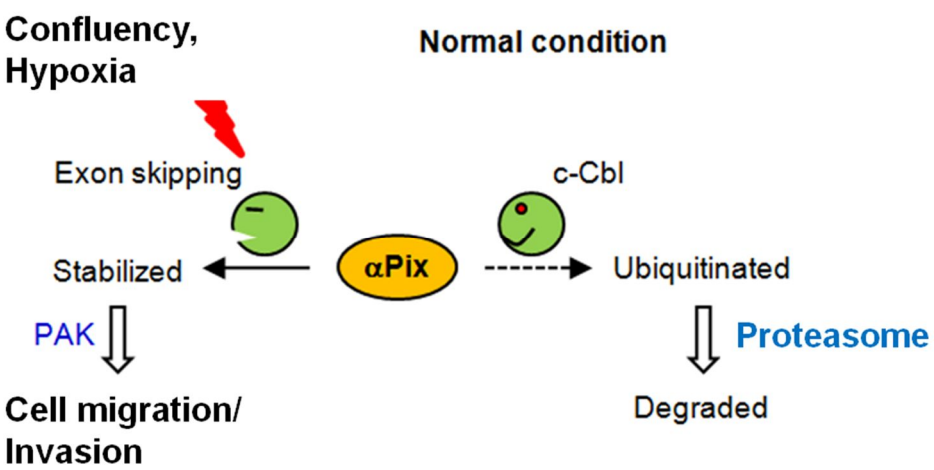
Notably, both the type I and type II c-Cbl exon skipping found in this study eliminated the exact length of exon-9 and both exon-9 and exon-10, respectively. Previously, it has been reported that genomic splice site mutations (*cis*-element mutation) cause deleterious exon skipping of c-Cbl (Dikic and Schmidt, 2007; Kales et al., 2010). However, no mutation was

found in the genomic DNA sequence, indicating that defects in *trans*-element are responsible for c-Cbl exon skipping. Moreover, exon skipping of c-Cbl could be observed only when cells were grown to confluence or under hypoxic conditions. These results suggest that the environment factors affect certain unknown *trans*-elements to catalyze c-Cbl exon skipping. How the environment factors make the *trans*-element to exert its splicing function is not at all known. Neither is known about the nature of *trans*-element involved in c-Cbl exon skipping nor what kind of defect is present in *trans*-element. Nevertheless, this study is the first report showing that certain defect (e.g., mutations) in *trans*-element could acquire a gain-of-function in splicing under environmental conditions, such as contact inhibition and hypoxic stress. In addition, c-Cbl exon skipping is clearly different from alternative splicing, because only one transcript of c-Cbl has so far been reported in the transcriptome database unlike typical alternative splicing, which generates two or more transcripts of one gene. In this respect, c-Cbl exon skipping is

entirely a new type of splicing, which would require a new term for future use. For this, I suggest “defective exon skipping” or “deleterious exon skipping.”

Figure 28. Model for tumor suppressive function of c-Cbl

Under normal conditions, c-Cbl of C6 and A172 cells ubiquitinates and destabilize αPix, preventing cell migration and thereby leading to cell growth arrest. Under conditions when cells are under confluent state and hypoxia, deleterious exon skipping occurs in c-Cbl transcripts for inactivation and destabilization of c-Cbl. As a consequence, αPix is stabilized and promotes cell migration and invasion for cells to acquire malignant behavior.


Confluency,
Hypoxia
Normal condition
Exon skipping
c-Cbl
Stabilized
αPix
Ubiquitinated
PAK
Proteasome
Cell migration/
Invasion
Degraded

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국문초록

c-Cbl은 RING 형태의 유비퀴틴 E3 ligase이며 성장인자수용체와 같은 타이로신인산화효소수용체를 유비퀴틴화-분해시켜 세포의 증식, 이동, 침투를 막는 기능을 한다. 그리고, 골수종양 환자들 사이에서 c-Cbl의 돌연변이가 자주 발견되고 있는 것으로 볼 때, c-Cbl은 종양억제 기능을 한다. αPix는 세포의 이동에 관여하는 단백질로 알려져 있다. αPix는 면역세포와 같이 매우 활발하게 증식하고 이동하는 세포에서만 발현되며 고착 세포에서는 발현되지 않는 것으로 알려져 있으나, 예외적으로 뇌종양과 같은 이동성과 침투성이 강한 고착 암세포에서는 발현된다. 본 논문에서는 c-Cbl이 αPix와 결합하여 유비퀴틴화-분해시키는 현상을 보였다. 아울러, 쥐의 뇌종양 세포인 C6, 사람의 뇌종양 세포인 A172, 그리고 몇몇 뇌종양 환자 조직에서, c-Cbl의 RING 도메인의 일부 엑손이 건너뛰어져 있는 것을 발견하였다. 이들 엑손이 건너뛰어진 c-Cbl은 αPix를 유비퀴틴화하지 못하였으며, 그로 인해 세포 내에 과량의 αPix가

누적되었다. 그리고, αPix의 누적에 의해 세포 내의 성장인자 신호가 강화되었으며, 그로 인해 세포의 이동성과 침투성 역시 증가되었다.

엑손 건너뛰기가 발생한 원인을 찾기 위해 인트론을 포함한 c-Cbl의 유전자 서열을 분석한 결과 유전자 상에는 돌연변이가 발견되지 않았다. 이에 엑손 건너뛰기가 발생한 유전자 일부분을 미니-유전자 형태로 만들어 뇌종양 세포주에 발현시킨 결과, C6와 A172 세포 내의 트랜스-요소의 문제로 인해 엑손 건너뛰기가 발생하는 것을 보였다. 또, 이 엑손 건너뛰기 현상은 세포 과밀화 현상 및 저산소증 신호와 관련되어 있다는 사실도 밝혔다. 이 결과로 볼 때, 뇌종양 세포 중 일부는 c-Cbl의 엑손 건너뛰기를 유발함으로써 세포 과밀화 현상과 저산소증을 극복하는 것으로 보이며, 이 현상은 뇌종양 세포의 악성형질과 밀접한 관계가 있는 것으로 보인다.

핵심어 : c-Cbl, αPix, 세포 이동, 유비퀴틴, 뇌종양, 엑손 건너뛰기, 세포 과밀화, 저산소증