

Identification of Novel Binding Partners for Caspase-6 Using a Proteomic Approach

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Apoptosis is the process of programmed cell death executed by specific proteases, the caspases, which mediate the cleavage of various vital proteins. Elucidating the consequences of this endoproteolytic cleavage is crucial to understanding cell death and other related biological processes. Although a number of possible roles for caspase-6 have been proposed, the identities and functions of proteins that interact with caspase-6 remain uncertain. In this study, we established a cell line expressing tandem affinity purification (TAP)-tagged caspase-6 and then used LC-MS/MS proteomic analysis to analyze the caspase-6 interactome. Eight candidate caspase-6-interacting proteins were identified. Of these, five proteins (hnRNP-M, DHX38, ASPP2, MTA2, and UACA) were subsequently examined by co-immunoprecipitation for interactions with caspase-6. Thus, we identified two novel members of the caspase-6 interactome: hnRNP-M and MTA2.

Keywords: Interactome, caspase-6, apoptosis, tandem affinity purification

Apoptosis (programmed cell death) is used in multicellular organisms in the development, maintenance of homeostasis, and removal of damaged cells [7]. Specialized proteases, termed caspases (for cysteine-aspartate proteases), are key proteins in the apoptotic process [14]. Caspases can be divided into two groups: initiator caspases (caspase-2, caspase-8, caspase-9, and caspase-10) and executioner caspases (caspase-3, caspase-6, and caspase-7) [18]. Inappropriate activation of the executioner caspases is prevented by their production as inactive procaspase dimers that must be cleaved by initiator caspases. Cleavage between the large and small subunits of the procaspase dimer enables a conformational change that brings the two active sites of the executioner caspase dimer together to create a functional mature protease [15]. Prior to cleaving the executioner caspases, monomeric caspase-8 must undergo oligomerization

to form dimers and possibly higher oligomers, and this oligomerization leads to autoprocessing and activation of the enzyme. The activated caspase-8 can then proteolytically process and activate the executioner caspases to amplify the death signal. In addition, activated caspase-8 can cleave Bid, a pro-apoptotic protein, which can then initiate the mitochondrial apoptotic pathway [11, 12]. Of the executioner caspases, caspase-7 and caspase-3 are closely related in terms of their primary sequence and substrate specificity [6, 8, 16, 19]. It is well known that caspase-3 cleaves a large set of substrates and plays a major role in apoptosis [10]. Meanwhile, the executioner caspase-6 possesses the distinctive feature that it can both activate [1] and be activated by caspase-3 and caspase-7 [9]. In addition, caspase-6 can process the initiator caspase-8 and caspase-10 [5, 9]. Thus, caspase-6 can act as both an initiator and an executioner

caspase. Recent literature showing that executioner caspases are differentially regulated and show distinct subcellular localizations during apoptosis [13] leads to the speculation that there may be caspase-6-specific substrates. Although a number of possible roles for caspase-6 have been proposed, the identities and functions of proteins that interact with caspase-6 remain uncertain. Thus, to better understand the biological roles of caspase-6 during apoptosis, in this study, we established a cell line that stably expresses tandem affinity purification (TAP)-tagged caspase-6. We then used LC-MS/MS proteomic analysis to analyze the caspase-6 interactome.

The cell line used in this study, termed TAP-CASP6, was established using HEK293 cells and a version of the TAP vector pNTAP containing the gene encoding caspase-6. To confirm the activity of the exogenous caspase in the cell line, the expression and cleavage of TAP-tagged caspase-6 and cleavage of PARP were analyzed by western blotting following treatment of the cells with either DMSO (control) or the apoptotic stimulus staurosporine (STS). Upon STS treatment, both PARP cleavage, which is an apoptotic hallmark, and the expression of exogenous caspase-6 were observed (Fig. 1A).

The key advantage of the TAP system is that the TAP tag is a dual affinity tag that enables the purification of the target protein (together with any binding partners) through two consecutive purification steps [2]. As the TAP tag used in this study contained a streptavidin-binding peptide (SBP) and a calmodulin-binding peptide (CBP), the initial

purification step was performed using streptavidin-coated beads, and the second purification step was done using calmodulin-coated beads. The TAP-tagged caspase bound to the resin tightly and was eluted efficiently in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 5 mM CaCl₂, 10 mM βME, 0.1% NP40, 10% glycerol, 2 mM biotin, and protease inhibitors. After each purification step, western blotting was performed using anti-SBP and anti-caspase-6 antibodies to determine the purity of the eluted protein. After the first purification step, the eluted protein was contaminated with endogenous caspase-6. However, after the second purification step, the endogenous protein had been completely eliminated (Fig. 1B).

The resulting eluents were precipitated and prepared by in-solution tryptic digestion for MS analysis. Then, the digested peptides were analyzed using an Agilent 1100 series nano-LC and LTQ-mass spectrometer. For data searching, the Mascot algorithm (Matrix Science, USA) was used to identify peptide sequences present in a protein sequence database. The database search criteria were as follows: taxonomy, *Homo sapiens* (NCBI database, downloaded on 12/11/01); fixed modification, carboxyamidomethylated at cysteine residues; variable modification, oxidized at methionine residues; maximum allowed missed cleavage, 2; MS tolerance, 1.2 Da; and MS/MS tolerance, 0.6 Da. Only peptides resulting from trypsin digest were considered. For reproducibility, three independent experiments were carried out, and candidates that were selected at least twice

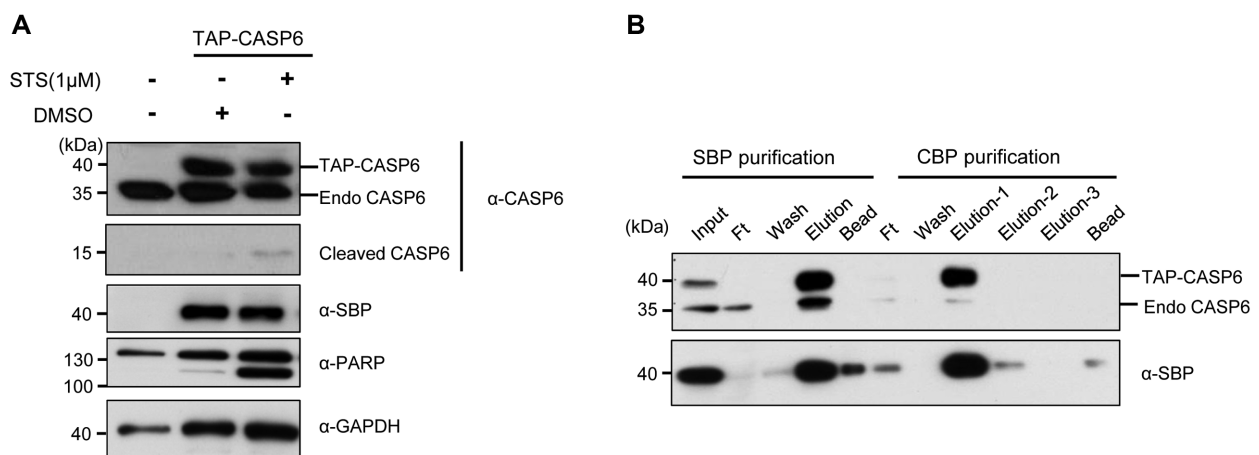


Fig. 1. Establishment of the TAP-CASP6 cell line and tandem affinity purification (TAP) of the caspase-6 interactome.

(A) The expression and cleavage of TAP-tagged caspase-6 (TAP-CASP6) and cleavage of PARP in TAP-CASP6 cells following treatment with either DMSO (control) or the apoptotic stimulus staurosporine (STS) were analyzed by western blotting using anti-PARP, anti-caspase-6, anti-streptavidin-binding peptide (SBP), and anti-GAPDH antibodies (α -PARP, α -CASP6, α -SBP, and α -GAPDH, respectively). GAPDH was used as the loading control. (B) Each fraction from the TAP procedure was analyzed by western blotting using both anti-caspase-6 and anti-SBP antibodies. CBP, calmodulin-binding peptide; Endo CASP6, endogenous caspase-6; Ft, flow-through fraction.

Table 1. List of proteins that interact with caspase-6 in TAP followed by LC-MS/MS.

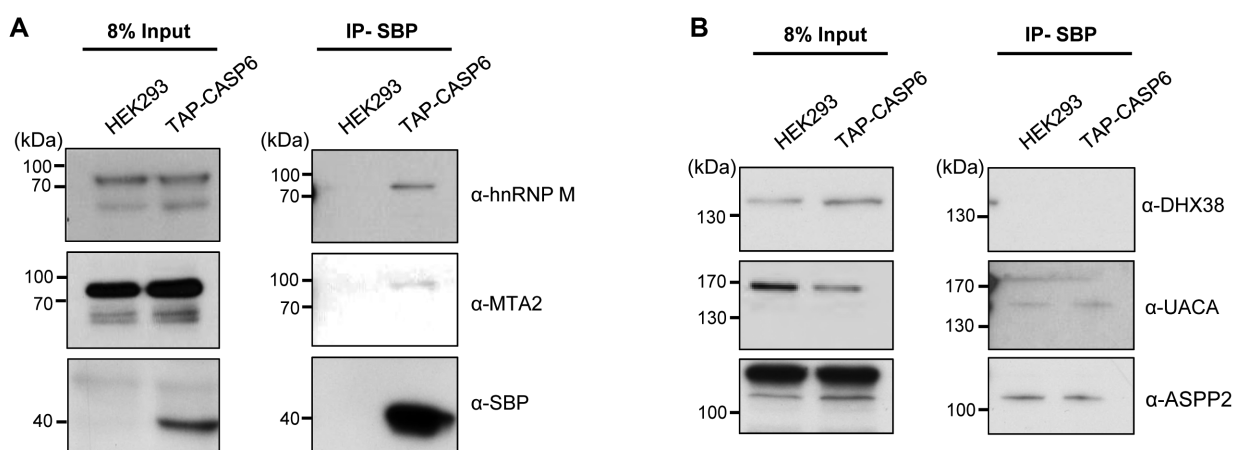
Swissprot number	Description (Taxonomy)	Coverage (%)	Mascot score ^a	Matched peptide
P55212 CASP6_HUMAN	Caspase-6	29.4	331	36
Q96JE9 MAP6_HUMAN	Microtubule-associated protein 6	36.4	73	16
P52272-2 HNRPM_HUMAN	Isoform 2 of heterogeneous nuclear ribonucleoprotein M	34.6	273	15
H0Y5B5 H0Y5B5_HUMAN	Protein polybromo-1 (Fragment)	8.5	55	22
Q9H1R3 MYLK2_HUMAN	Myosin light chain kinase 2; skeletal/cardiac muscle	8.7	82	13
O94776 MTA2_HUMAN	Metastasis-associated protein MTA2	8.1	41	14
F5H2B9 F5H2B9_HUMAN	Uveal autoantigen with coiled-coil domains and ankyrin repeats	16.5	43	16
Q92620 PRP16_HUMAN	Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	9.7	63	12
Q13625 ASPP2_HUMAN	Apoptosis-stimulating of p53 protein 2	7.4	73	10

^aThe respective protein scores have confidence region and displayed along with the numbers with a 95% significance.

are listed in Table 1. As a control, the eluents from cells expressing the empty pNTAP vector were analyzed as well, and candidates that were found in those eluents were excluded. In addition, candidates with less than 95% significance were eliminated. As a result, eight proteins were identified as possibly interacting with caspase-6 (Table 1).

To verify the interactions of these candidate proteins with caspase-6, we performed co-immunoprecipitation experiments using an anti-SBP antibody and commercially available antibodies for the candidate proteins as follows. The cell extracts from either HEK293 cells or TAP-CASP6 cells were prepared as described previously [4] and immunoprecipitated using streptavidin-coated agarose

beads (Agilent). After washing the beads three times with a buffer containing 20 mM HEPES (pH 7.5), 0.5% NP40, 200 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, and a protease inhibitor, the resulting complex was eluted from the beads by boiling and then analyzed by immunoblotting. As shown in Fig. 2A, caspase-6 co-immunoprecipitated with hnRNP-M and MTA2, although ASPP2, UACA, and DHX38 did not show any interaction with caspase-6 (Fig. 2B). Whole cell lysates (8% input) were also analyzed by western blotting using anti-hnRNP-M, anti-MTA2, anti-DHX38, anti-UACA, anti-ASPP2, and anti-SBP antibodies, respectively. These results confirmed that hnRNP-M and MTA2 are binding partners of caspase-6.

**Fig. 2.** Verification of caspase-6 binding partners by co-immunoprecipitation.

Western blots of whole cell lysates from HEK293 control cells or cells expressing tandem affinity purification (TAP)-tagged caspase-6 (TAP-CASP6) before (8% input) and after immunoprecipitation using streptavidin-coated beads (IP - SBP). (A) Western blot using anti-hnRNAP-M, anti-streptavidin-binding peptide (SBP), and anti-MTA2 antibodies (α -hnRNAP-M, α -SBP, and α -MTA2, respectively). (B) Western blot using anti-DHX38, anti-UACA, and anti-ASPP2 antibodies (α -DHX38, α -UACA, and α -ASPP2, respectively).

In this study, by using TAP purification followed by LC-MS/MS to analyze the caspase-6 interactome, we identified MTA2 and hnRNP-M as proteins that interact with caspase-6. It is known that cell stress induced by STS causes the nuclear translocation and activation of caspase-6 in primary striatal neurons [20]. MTA2 and hnRNP-M are known to reside in the nucleus. Thus, the interaction of these two proteins with caspase-6 could occur in the nucleus after the onset of apoptosis. It has been shown that MTA2 binds the tail domain of histone H3 and is involved in metastasis. MTA2 knockdown in human SGC-7901 and AGS gastric cancer cells significantly inhibited migration and invasion *in vitro*, and attenuated xenograft growth and lung metastasis in a nude mice model [22]. The interaction between MTA2 and caspase-6 indicates that caspase-6 may play a role in the regulation of MTA2 during apoptosis. It is intriguing that a putative caspase-6 interacting partner, polybromo-1/BAF180, which was originally identified as a component of the PBAF chromatin remodeling complex, has also been shown to bind to acetylated histones [21]. Thus, caspase-6 may, in some cases, associate with histone-binding proteins. In addition, polybromo-1 has been known to occupy the promoter region of p21, a p53 target gene, and thus regulate p21 transcription [3]. Thus, it is feasible that the interaction between caspase-6 and polybromo-1 may affect the transcription of p21. In the liver, carcinoembryonic antigen binds to hnRNP-M at the surface of Kupffer cells and causes activation and production of pro- and anti-inflammatory cytokines, including IL-1, IL-10, IL-6, and TNF- α . These cytokines affect the upregulation of adhesion molecules on the hepatic sinusoidal endothelium and protect tumor cells against cytotoxicity by nitric oxide and other reactive oxygen radicals. This has implications for the control of tumor cell implantation and survival in the liver [17], suggesting that caspase -6 affects the function of hnRNP-M in tumor cell survival.

In conclusion, we used the TAP system and proteomic analysis to screen for caspase-6-interacting proteins during apoptosis. We isolated eight candidate proteins, two of which are known to be localized to the nucleus and were confirmed as binding partners of caspase-6 by co-immunoprecipitation. Our results may provide insight into the function of caspase-6 in the nucleus during apoptosis.

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