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Identification of the recognition sequence and target proteins for DJ-1 protease

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ABSTRACT

DJ-1 is a product for familial Parkinson's disease gene and for an oncogene and plays a role in anti-oxidative stress reaction. DJ-1 is also a cysteine protease, but the characteristics of DJ-1 protease remain unclear. In this study, we identified the recognition sequence for DJ-1 protease by using recombinant DJ-1 and a peptide library. Protease activity of DJ-1 lacking C-terminal α-helix (DJ-1ΔH9) was stronger than that of full-sized DJ-1, and the most susceptible sequence digested by DJ-1ΔH9 was valine-lysine-valine-alanine (VKVA) under the optimal conditions of pH 5.5 and 0 mM NaCl. Divalent ions, especially Cu^{2+}, were inhibitory to DJ-1's protease activity. c-*abl* oncogene 1 product (ABL1) and kinesin family member 1B (KIF1B) containing VKVA were digested with DJ-1ΔH9.

Keywords: DJ-1, protease, biochemistry

1. Introduction

DJ-1 was first identified by our group as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated *ras* [1] and was later found to be a causative gene for a familial form of Parkinson's disease [2]. DJ-1 has multiple functions, including transcriptional regulation [3-6], anti-oxidative stress function [7-9], mitochondrial regulation [8, 10-12], and functions as a chaperone [13] and protease [14-16]. The structure of DJ-1 is similar to that of cysteine protease from *Pyrococcus horikoshii*, but C-terminal α-helix 9 blocks a catalytic domain of protease [17]. DJ-1 is cleaved at the C-terminal region under oxidative stress conditions *in vitro* and in cells [16, 18], and a C-terminally cleaved form of DJ-1 enhances cytoprotective action against oxidative stress-induced apoptosis [16]. Transthyretin, a causative protein in familial amyloidotic polyneuropathy, is degraded in cells transfected with full-sized DJ-1 and *in vitro* by recombinant DJ-1 lacking α-helix 9 (DJ-1ΔH9), and mutation of C106 in DJ-1 results in loss of its protease activity [15]. These results suggest that α-helix 9 of DJ-1 is opened in cells by oxidative stress that causes pathological phenomena such as neurodegenerative diseases. Identification of the recognition sequence of DJ-1 protease and of the protein(s) that opens α-helix 9 of DJ-1 in cells will lead to elucidation of the physiological role of DJ-1 protease.

In this study, we identified the recognition sequence of DJ-1 by using recombinant DJ-1ΔH9 and a peptide library, and c-*abl* oncogene 1 product (ABL1) and kinesin family member 1B (KIF1B) were digested by DJ-1ΔH9.

2. Materials and methods

2.1. Plasmids and purification of DJ-1

Plasmids harboring GST-tagged wild-type DJ-1, C106S DJ-1, DJ-1ΔH9 and C106S DJ-1ΔH9 were described previously [15]. GST-tagged DJ-1s were expressed in and purified from *E. coli* and digested with PreScission protease (GE Bioscience, Little Chalfont, UK) to cleave off GST

as described previously [15] To construct deletion mutants lacking VKVA sequences of ABL1 and KIF1B, following PCR primers were used. ABL1ΔVKVA-S: 5'-GGAGAACCACTTGGATTTTGGCCTGAGC-3', ABL1ΔVKVA-AS: 5'-GCTCAGGCCAAAATCCAAGTGGTTCTCC-3', KIF1BΔVKVA-S: 5'-GGAGCCTCAGTCCGGGTAAGGCC-3', and KIF1BΔVKVA-AS: 5'-GGCCTTACCCGGACTGAGGCTCC-3'. PCR was carried out using pTOPO-ABL1 and pTOPO-KIF1B as templates and primers above. Resultant PCR products were digested with *Dpn*I and used for transformation into *E. coli*.

2.2. Screening of the peptides digested with DJ-1

First screening: Ten μM fluorescence-quenching substrate library FRETS-25Xaa Series (Peptide Institute, Osaka, Japan) was reacted with 15 μg of recombinant DJ-1 in a buffer containing 0.5 mM acetate (pH 5.5) and 0.2 mM $CaCl_2$ at 37°C for 8-21 hrs and its fluorescence intensity was measured using a fluorometer (GloMax, Promega, Madison, WI, USA). Second screening: Three candidates of DJ-1 target sequences that had been identified in the first screening (FRETS-25Val, FRETS-25Ile and FRETS-25Ala) were reacted with 100 μM FRETS-25Xaa Series and 67.5 μg of DJ-1ΔH9 in the same buffer as above at 37°C for 8 or 7 hrs. Then, a final concentration of 0.5 M EDTA (pH 8.1) was added to each reaction mixture, and the reaction mixtures were frozen in liquid nitrogen and subjected to LC-MS analysis to determine amino acids corresponding to Yaa and Zaa sites as described previously [19].

2.3. Digestion of target protein candidates for DJ-1 protease in vitro

cDNAs encoding target proteins for DJ-1 protease were cloned into pcDNA3. ^{35}S-labeled proteins were then synthesized *in vitro* using reticulocyte lysate of the TNT transcription-translation coupled system (Promega) and reacted with 1 μg of recombinant DJ-1 in a buffer containing 0.5 mM acetate (pH 5.5) and 0.2 mM $CaCl_2$ at 37°C for 4 hrs. Bovine serum albumin (BSA) as a negative control was also reacted with ^{35}S-labeled proteins. Labeled

proteins were then separated on SDS-containing polyacrylamide gels and visualized by fluorography.

2.7. Statistical analyses

Data are expressed as means ± S.E. Statistical analyses were performed using the Tukey-Kramer test.

3. Results

3.1. Identification of amino acid sequences digested by DJ-1

Since a C-terminal α-helix 9 in DJ-1 blocks the putative catalytic region of DJ-1 protease and since we have shown that recombinant DJ-1 lacking α-helix 9 (DJ-1ΔH9) digested transthyretin *in vitro* and that this activity was diminished by substitution of an amino acid from cysteine to serine at 106 (C106S) [15], 4 recombinant DJ-1s were expressed in and purified from *E. coli* (Fig. 1A) and used in this study. BSA was also used as a negative control. Fluorescence-quenching substrate library FRETS-25Xaa Series possesses a fluorescence group (Nma) and a quenching group (Dnp), and Nma's emission of florescence light is not activated by Dnp (Fig. 1B). When peptides in the library are digested with protease, fluorescence intensity of Nma is increased, enabling measurement of protease activity. The Xaa (P1) site contains 19 different amino acids other than cysteine, and Yaa (P2) and Zaa (P3) sites contain 5 kinds of amino acids with different characteristics. Each Xaa series is therefore a mixture of 25 kinds of peptides, and the whole library contains 475 kinds of peptides (Fig. 1B).

To determine amino acids in an Xaa site, FRETS-25Xaa Series was reacted with 4 recombinant DJ-1s and BSA for 21 hrs and its fluorescence was measured. Results for valine (Val), isoleucine (Ile), alanine (Ala) and proline (Pro) in the Xaa site are shown as examples, in which Pro in the Xaa site of the peptide was not digested with DJ-1 (Fig. 2A). While wild-type DJ-1, C106S DJ-1, C106SΔH9 DJ-1 and BSA showed no or little protease activity against FRETS-25Xaa Series, DJ-1ΔH9 digested FRETS-25Xaa Series with Val, Ile and Ala in the Xaa

sites in a time-dependent manner. A summary of DJ-1 protease activities using various amino acids in the Xaa sites in the library is shown in Fig. 2B. Of 19 amino acids, Val, Ile and Ala, which are categorized into nonpolar amino acids, in the Xaa sites of the peptide were found to be good substrates for protease activity of DJ-1ΔH9. As the second screening, FRETS-25Val, -25Ile and -25Ala were reacted with DJ-1ΔH9 for 4 hrs, and amino acids in Yaa and Zaa of FRETS-25Val, -25Ile and -25Ala were determined by using LC-MS. Ratio of peak area for FRETS-25Val in MS profiles versus enzyme digestibility was plotted (Fig. 2C), and a summary of all of the results is shown in Table 1. In the case of FRETS-25Val, for instance, the order of sequence digestibility to DJ-1ΔH9 is VKV > VPV > RYV > FKV > RKV. DJ-1 protease digested the peptide bond between P1 amino acids and Ala.

3.2. Characteristics of DJ-1 protease

Biochemical characterization of DJ-1 protease was then carried out using VKVA peptide (D-A2pr(Nma)-GVKVAFPK(Dnp)rr) as a substrate and recombinant DJ-1s. Protease activity of DJ-1ΔH9 appeared at pH 5.5-6.0 and disappeared at pH 7.4 and 9.5 (Fig. 3A), indicating that a weak acidic condition is optimal. DJ-1 protease activity was decreased in a salt-dependent manner and the concentration of 0 mM NaCl was maximal (Fig. 3B). Also, DJ-1 protease did not require divalent ions such as Ca^{++}, Mg^{++} and Cu^{++}, and even a low concentration of Cu^{++} diminished protease activity (Fig. 3C). Kinetic analysis of the enzymatic reaction showed that the reaction of DJ-1ΔH9 protease activity obeyed the Michaelis-Menthene formula in which substrate concentration and reaction rate were placed on horizontal and longitudinal axes, respectively (Fig. 3D). Molecular activity and Michaelis-Menthen constant (*k*cat and *K*m values, respectively) were then calculated. The *k*cat value is represented as substrate concentration/enzyme concentration x seconds, and *k*cat of DJ-1ΔH9 protease was calculated to be 1.02×10^{-4} (S^{-1}) (Fig. 3F). After the Lineweaver-Burk plot was made, *K*m was calculated to be 40.1 μM, which is $1/10^5$ of that of trypsin, a strong protease (Figs. 3E and 3F, respectively). These results indicate that although protease activity of DJ-1ΔH9 is not strong, DJ-1ΔH9 has

biochemical characteristics for protease.

Since Pfp1, whose crystal structure is similar to that of DJ-1, is a class of the cysteine protease and since a cysteine substitution mutant of DJ-1, C106S DJ-1, lost protease activity (Fig. 2A), it is thought that DJ-1ΔH9 is also a cysteine protease. To ascertain this, two inhibitors for cysteine protease were reacted with DJ-1ΔH9. E-64 is a specific inhibitor of cysteine protease and inhibits protease activity of papain. N-ethylmaleimide (NEM) nonselectively inhibits cysteine protease. As shown in Figs. 3G and 3H, protease activity of DJ-1ΔH9 was inhibited by NEM, suggesting that, like Pfpi, DJ-1ΔH9 is a cysteine protease.

3.3. Identification of candidate proteins for DJ-1ΔH9 protease

A search for proteins that contain recognition sequences of DJ-1 protease was made by using the BLAST search against 3 amino acids shown in Table 1, and more than 2000 proteins were found. Since it is not possible to examine digestibility of all of these proteins, we focused on recognition sequence-containing proteins that interacted with DJ-1 such as DJBP, HIPK1α, HIPK1β, Topors, PTEN and tyrosine hydroxylase or that play roles in pathogenesis of cancer such as ABL1 and of Parkinson's disease (PD) such as LRRK2 and in mitochondrial regulation such as KIF1B, POMC and UCP1 [3, 4, 7, 20-27]. These proteins were synthesized *in vitro* using reticulocyte lysates and reacted with DJ-1ΔH9, wild-type DJ-1and C106SΔH9. Of 14 proteins examined, only ABL1 and KIF1B were cleaved by DJ-1ΔH9 but not by wild-type DJ-1and C106SΔH9, and the results for ABL1, KIF1B and HIPK1α as a negative example are shown in Fig. 4A. When ABL1 and KIF1B were reacted with various amounts of DJ-1ΔH9, they were digested with DJ-1ΔH9 in a dose-dependent manner (Fig. 4B). ABL1 and KIF1B possess a VKVA sequence, the most digestible sequence for DJ-1ΔH9 protease, at amino acid numbers 398 and 6 in the kinase domain and in the motor domain, respectively (Fig. 4D). When these sites were digested with DJ-1ΔH9, ABL1 and KIF1B should be cleaved to two fragments with approximately 44 and 82 kDa and to those with approximately 0.7 and 126 kDa, respectively, and sizes of cleaved bands observed in Figs. 4A and 4B are well matched. A 126-kDa band of KIF1B cleaved by

DJ-1ΔH9 was observed on bands that appeared in all the lanes. These bands may be translated from internal methionine of KIF1B or bands cleaved by protease present in reticulocyte lysates. The KIF1B fragment of 0.7 kDa was run through gels and only a band with 126 kDa was observed. To confirm whether a VKVA sequence in ABL1 and KIF1B is a target sequence for DJ-1 protease, ABL1 and KIF1B mutants lacking the VKVA sequence were constructed and reacted with wild-type DJ-1, DJ-1ΔH9 and C106SΔH9. As shown in Fig. 4C, these mutants were not cleaved by DJ-1ΔH9. The reduced level of full-sized KIF1B mutants lacking the VKVA sequence was reproducibly observed. This may be due to conformational change of KIF1B mutant that resulted in low translational efficiency. These results suggest that ABL1 and KIF1B are candidate proteins for DJ-1 protease.

4. Discussion

In this study, we first identified recognition sequences for DJ-1 protease and found that VKVA, VKIA and VYAA were preferentially cleaved by DJ-1 lacking C-terminal α-helix 9 (DJ-1ΔH9) *in vitro*, and wild-type, C10S DJ-1 and C106SΔH9 DJ-1 had no or little protease activity (Figs. 1 and 2, Table 1). DJ-1ΔH9 protease has biochemical characteristics of protease (Fig. 3), and ABL1 and KIF1B were identified to be candidate proteins for DJ-1 protease (Fig. 4). Preferred amino acids of the P1 site for DJ-1 protease are Val, Ile and Ala, which are non-polar amino acids, and these phenomena are similar to those for Pfpi [28]. Optimal pH for DJ-1 protease was 5.5-6.0 and its activity decreased at more alkaline pH (Fig. 3). These results imply two possibilities: One is that DJ-1 protease works under oxidative stress conditions that induce acidic regions locally in cells, and the other possibility is that DJ-1 protease works in some organelles in which relatively low pH is maintained. Such organelles include the endosome, lysosome, Golgi apparatus and synaptic vesicle. Of these organelles, the lysosome contains many proteases whose optimal pH is around 5. It is possible that DJ-1 translocates to the lysosome to explore its protease activity under some stress conditions. Cu^{++} strongly inhibited DJ-1ΔH9 protease activity (Fig. 3C). Cu^{++} is known to be a risk factor for Parkinson's disease and has been reported to bind

to DJ-1 [29]. It is therefore thought that Cu^{++} binds to a near active site of DJ-1 protease, thereby inhibiting its activity.

ABL1 and KIF1B were identified as target proteins for DJ-1 protease (Fig. 4). ABL1 is a proto-oncogene product and works as a non-receptor-type tyrosine kinase in cell differentiation, cell growth and cell adhesion, and its overexpression or structural abnormality results in onset of cancer [30]. Augmented expression and activity have also been reported in patients with Alzheimer's disease and amyotrophic lateral sclerosis [31-33], and overexpression of ABL1 leads to apoptosis induction [33]. Furthermore, it has been reported that ABL1 phosphorylates Parkin, a causative gene product of familial Parkinson's disease (Park 2), resulting in inactivation of its ubiquitin ligase activity [34]. It is therefore possible that DJ-1 degrades ABL1 to inhibit its kinase activity, thereby maintaining neuroprotective activity of Parkin. KIF1B is a motor protein belonging to the Kinesin family of proteins and has two splicing isoforms, KIF1Bα and KIF1Bβ [25, 35, 36]. KIF1Bα and KIF1Bβ transport mitochondria in nerve cells and the endoplasmic reticulum, respectively, and possess a VKVA sequence. It is possible that DJ-1 inhibits mitochondrial transport through cleavage of KIF1Bα.

We have shown that transthyretin is cleaved by DJ-1ΔH9 *in vitro* and that transthyretin is cleaved by transfection of full-sized DJ-1 in cells [15]. Chen et al. reported that DJ-1 in dopaminergic cells undergoes C-terminal cleavage in response to mild oxidative stress, and a C-terminally cleaved form of DJ-1 with activated protease activity enhances cytoprotective action against oxidative stress-induced apoptosis [16]. These results suggest that the C-terminal sequence of DJ-1 is opened under some stress conditions in cells. Although the physiological function of DJ-1 protease is still not known, the present findings will contribute to elucidation of DJ-1 function against stresses.

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Legends of figures

Fig. 1. DJ-1 mutants and FRETS-25Xaa Series.

A. Schematic drawing of recombinant DJ-1s used in this study (left panel). Purified DJ-1 proteins were separated on 12% polyacrylamide gel and stained with coomassie brilliant blue (right panel).

B. Schematic drawing of FRETS-25Xaa Series.

Fig. 2. Identification of recognition sequences for DJ-1 protease.

A. Amino acids in the P1 site of FRETS-25Xaa Series were identified using various DJ-1s shown in Fig. 1A and BSA. Results for Val, Ile, Ala and Pro in the P1 site are shown.

B. Summary of the results of experiments described in the legend for Fig. 2A.

C. Amino acids in P2 and P3 sites of FRETS-25Val were identified as described in Materials and methods.

Fig. 3. Biochemical characterization of DJ-1 protease.

A-C. Optimal conditions of pH (A), NaCl concentration (B) and concentration of divalent ions (C) were examined for valine, lysine and valine (VKV) in P1, P2 and P3 sites of FRETS-25Xaa using DJ-1ΔH9.

D, E. Michaelis-Menten kinetics (D) and Lineweaver-Burk plot (E) were drawn after reaction of DJ-1ΔH9 with VKV in P1, P2 and P3 sites of FRETS-25Xaa.

F. *k*cat and *K*m were calculated from the results shown in Figs. 3D and 3E.

G, H. DJ-1ΔH9 was reacted with VKV in P1, P2 and P3 sites of FRETS-25Xaa and various concentrations of E-64 (G) and N-ethylmaleimide (H), and protease activity of DJ-1ΔH9 is shown.

Fig. 4. Identification of ABL1 and KIF1B as target proteins for DJ-1 protease.

A. ^{35}S-labeled ABL1, KIF1B and HIPK1α were synthesized *in vitro* using reticulocyte lysates, reacted with wild-type, ΔH9 and C106SΔH9 DJ-1 and BSA, and separated on polyacrylamide gels. Proteins were then visualized by fluorography.

B. ^{35}S-labeled ABL1 and KIF1B were reacted with various concentrations of wild-type and ΔH9 DJ-1 and visualized by fluorography.

C. ^{35}S-labeled ABL1, ABL1ΔVKVA, KIF1B and KIF1BΔVKVA were reacted with wild-type, ΔH9 and C106SΔH9 DJ-1 and subjected to fluorography. Full-sized ABL1 and KIF1B, and large cleaved products of ABL1 and KIF1B are shown by arrows.

D. Schematic drawing of structures of ABL1 and KIF1B.

Table 1. Relative hydrolysis rate of DJ-1ΔH9 for FRETS substrates.

FRETS-25Val		FRETS-25Ile		FRETS-25Ala	
P3P2P1	Relative rate	P3P2P1	Relative rate	P3P2P1	Relative rate
VKV	100 %	VKI	100 %	VYA	100 %
VPV	64	RYI	64	VKA	59
RYV	54	VPI	50	RYA	50
FKV	52	RKI	49	VPA	34
RKV	51	FKI	47	RIA	33

Digestibility of amino acids in P1, P2 and P3 sites for DJ-1ΔH9 protease is shown.

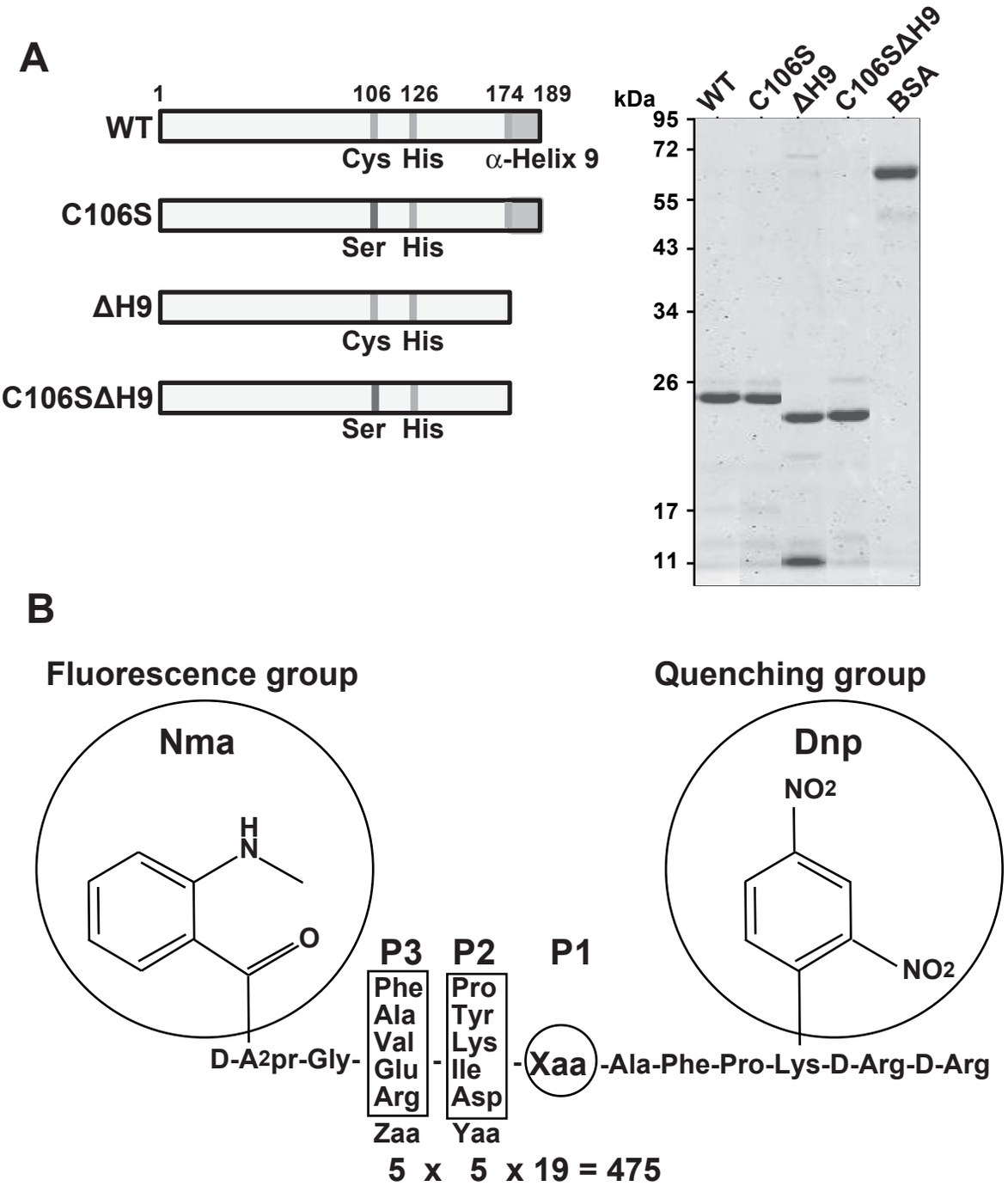
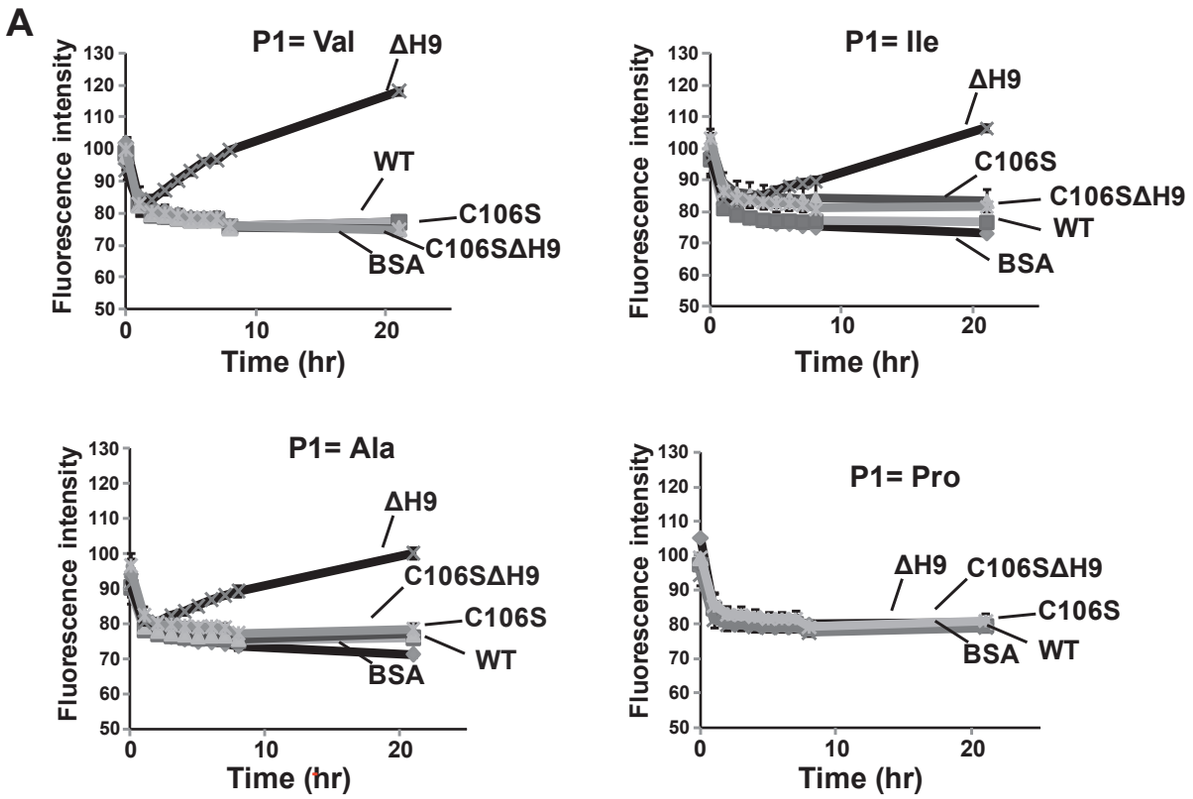
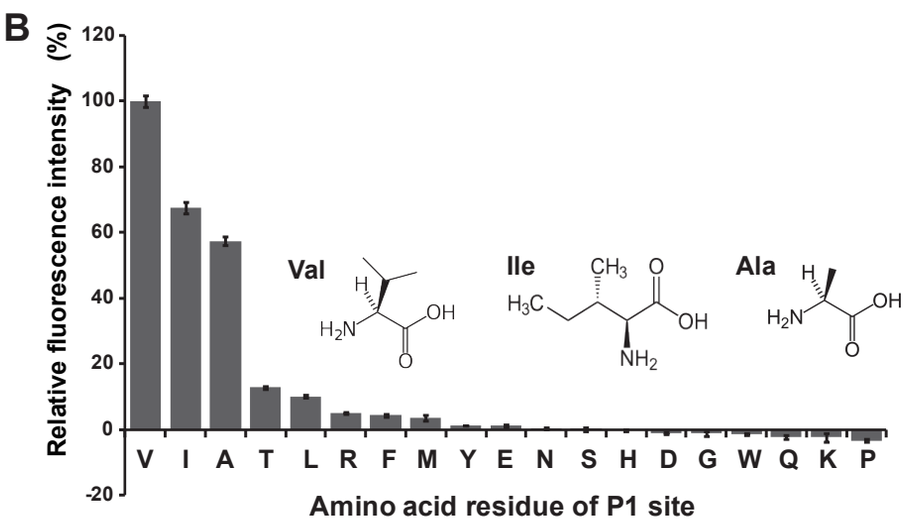

A
1
106 126
174 189
WT
Cys His
α-Helix 9
C106S
Ser His
ΔH9
Cys His
C106SΔH9
Ser His
kDa
WT
C106S
ΔH9
C106SΔH9
BSA
95
72
55
43
34
26
17
11
B
Fluorescence group
Quenching group
Nma
Dnp
NO2
NO2
H
N
O
P3
P2
P1
Phe
Ala
Val
Glu
Arg
Pro
Tyr
Lys
Ile
Asp
Xaa
D-A2pr-Gly-
-Ala-Phe-Pro-Lys-D-Arg-D-Arg
Zaa
Yaa
5 x 5 x 19 = 475

Fig. 1


A
P1= Val
ΔH9
WT
C106S
C106SΔH9
BSA
P1= Ile
ΔH9
C106S
C106SΔH9
WT
BSA
P1= Ala
ΔH9
C106SΔH9
C106S
WT
BSA
P1= Pro
ΔH9
C106SΔH9
C106S
BSA
WT
Fluorescence intensity
Time (hr)


B
Relative fluorescence intensity (%)
Val
Ile
Ala
V I A T L R F M Y E N S H D G W Q K P
Amino acid residue of P1 site

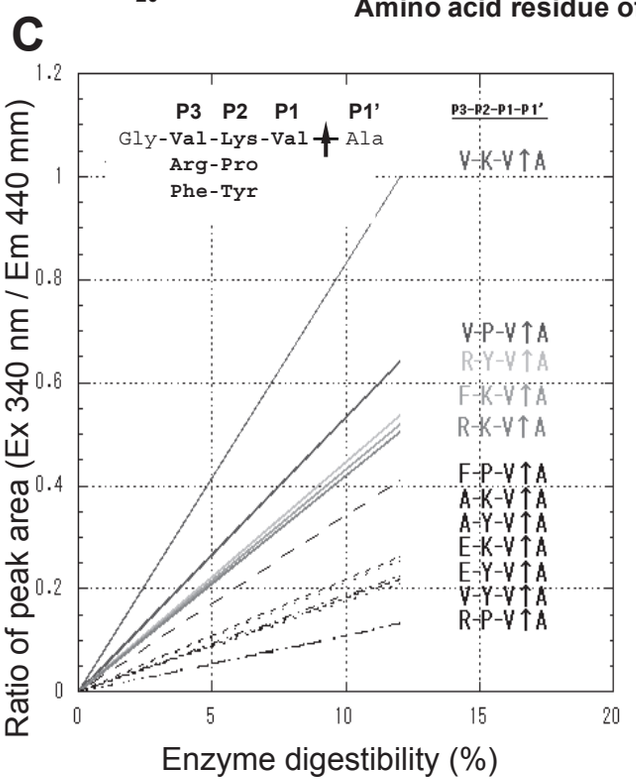
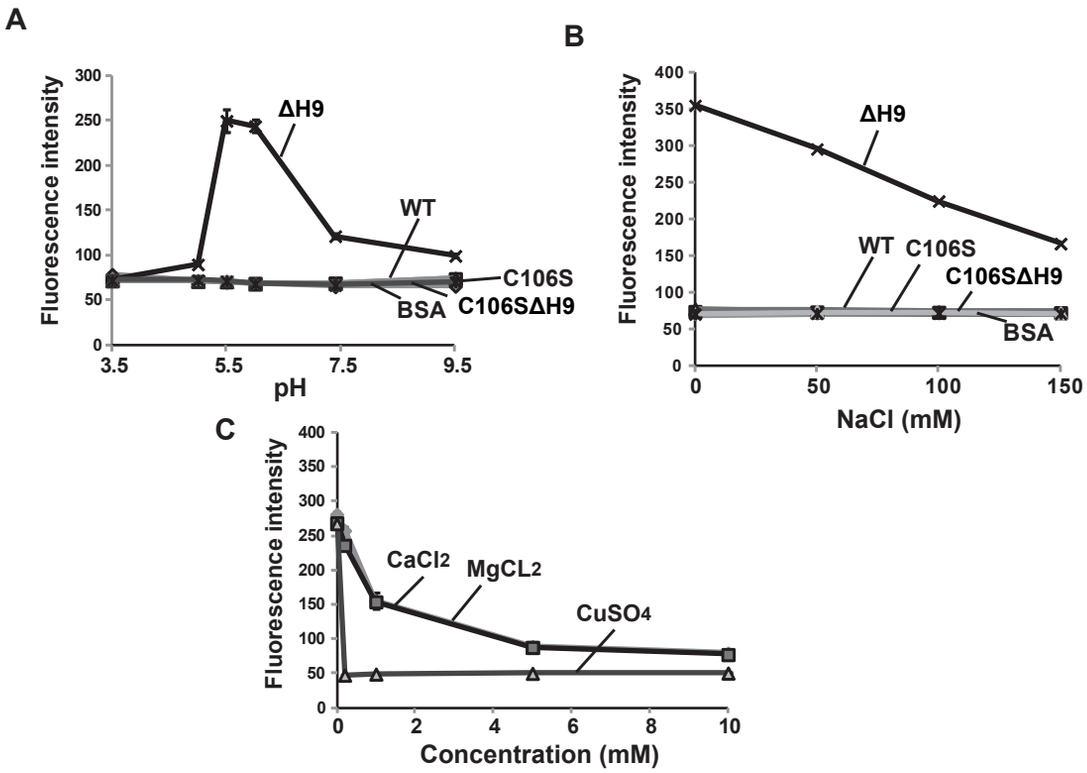
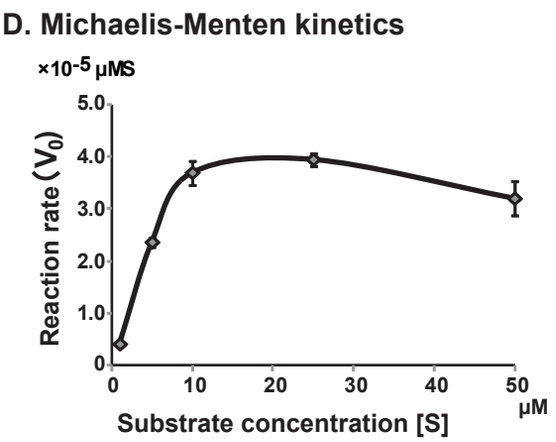
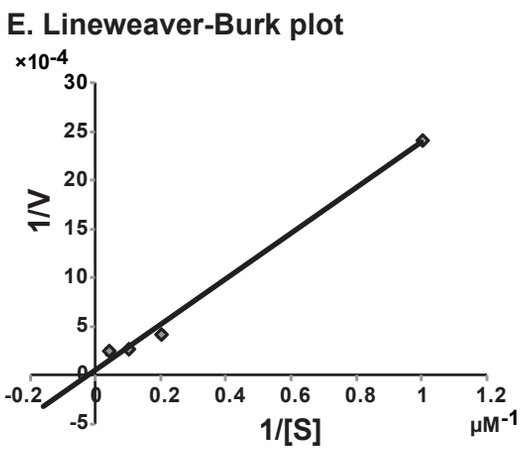

C
P3 P2 P1 P1'
Gly-Val-Lys-Val Ala
Arg-Pro
Phe-Tyr
P3-P2-P1-P1'
V-K-V↑A
V-P-V↑A
R-Y-V↑A
F-K-V↑A
R-K-V↑A
F-P-V↑A
A-K-V↑A
A-Y-V↑A
E-K-V↑A
E-Y-V↑A
V-Y-V↑A
R-P-V↑A
Ratio of peak area (Ex 340 nm / Em 440 mm)
Enzyme digestibility (%)

Fig. 2

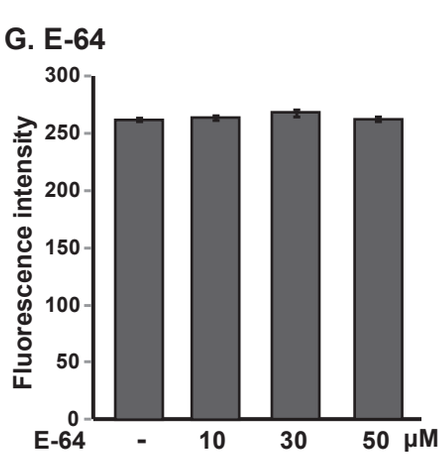






F

	*k*cat (S^{-1})	*K*m (μM)	*k*cat/*K*m ($M^{-1} S^{-1}$)
DJ-1ΔH9	1.02 x 10^{-4}	40.1	2.5
Trypsin			2.5 x 10^{5}



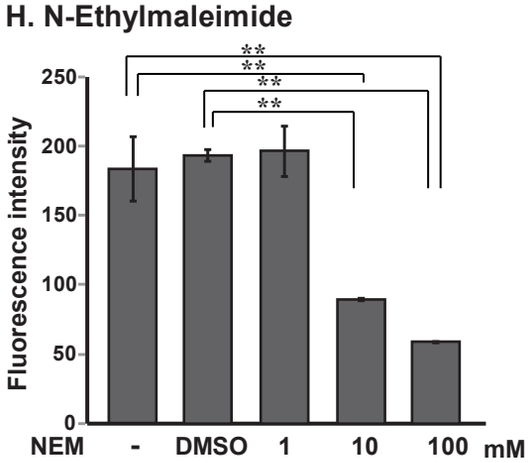


Fig. 3

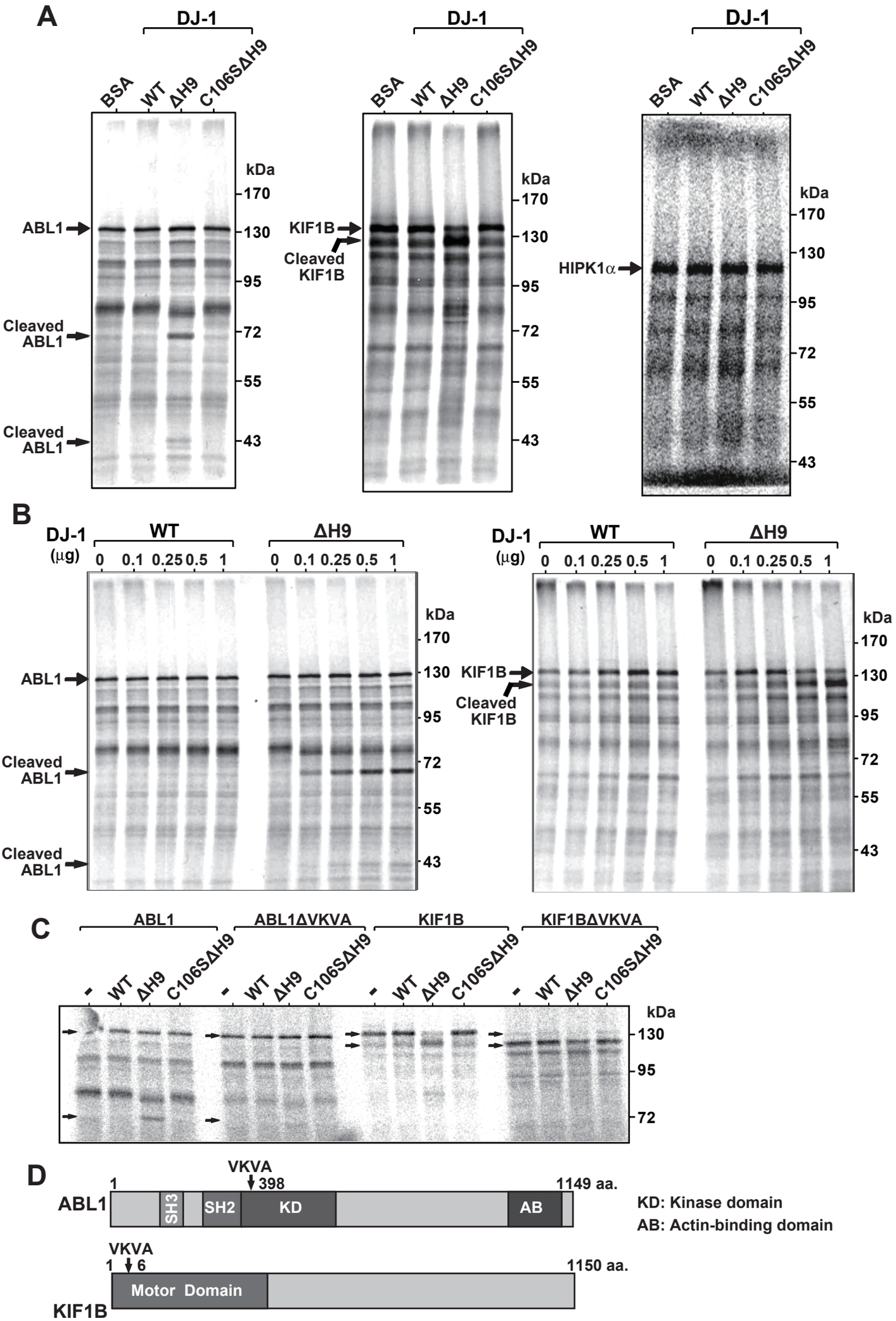

A
DJ-1
BSA
WT
ΔH9
C106SΔH9
kDa
170
130
95
72
55
43
ABL1
Cleaved ABL1
Cleaved ABL1
KIF1B
Cleaved KIF1B
HIPK1α
B
DJ-1 (μg)
WT
ΔH9
0
0.1
0.25
0.5
1
ABL1
Cleaved ABL1
Cleaved ABL1
KIF1B
Cleaved KIF1B
C
ABL1
ABL1ΔVKVA
KIF1B
KIF1BΔVKVA
-
WT
ΔH9
C106SΔH9
D
VKVA
398
1
1149 aa.
ABL1
SH3
SH2
KD
AB
KD: Kinase domain
AB: Actin-binding domain
VKVA
1
6
1150 aa.
Motor Domain
KIF1B

Fig. 4