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SHORT COMMUNICATION

UBIQUITIN AND PROTEASOMES ARE INVOLVED IN THE DEGRADATION OF CYTOSOLIC DOPPEL PROTEIN

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ABSTRACT

When HEK293 cells were transfected with a plasmid containing CMV promoter driven human wild-type *PRND* gene, the doppel protein was diffusely observed at the cytoplasm as seen in cancer cells. The transfected cells incubated with proteasome inhibitor N-acetyl-leucinal- leucinal- norleucinal (ALLN), resulted in apparent accumulation of the doppel protein. Furthermore, an alpha helix domain of the doppel protein appeared to be coimmunoprecipitated with ubiquitin related HSP70 protein. These findings suggested that the cytosolic doppel protein chaperoned with HSP70 could be subjected to ubiquitin proteasome degradation system.

INTRODUCTION

Doppel gene (*PRND*) is a paralogue of the mammalian Prion gene (*PRNP*). Similarly to the prion protein, doppel (Dpl) is thought as a glycosyl phosphatidylinositol anchored protein. When overexpressed, the Dpl is neurotoxic and causes a neurological disease, although Dpl shares significant biochemical and structural homology with the normal prion protein, suggesting that they are related biological function. The Dpl can induce Purkinje cell death by nonapoptotic mechanisms, even in the absence of proapoptic Bax protein (Dong *et al.,* 2007). Strikingly, the neurotoxicity of Dpl is counteracted and prevented by the normal prion protein. Antagonistic functions for them were thus indicated from genetic and biochemical evidences. On the other hand, Dpl appears to have an essential function in male spermatogenesis (Qin *et al.,* 2006), however, the function of Dpl has not been completely established. Ectopic expression of Dpl was found in glial and hematological cancers. Moreover, the level of Dpl accumulation seems associated with astrocytoma malignancy grades (Comincini *et al.,* 2007). The Dpl from the astrocytoma cells is found in the cytoplasm (Comincini *et al.,* 2006), whereas in the microsome fraction from normal cells. The cytosolic soluble Dpl protein in astrocytoma-derived cells exhibited some biochemical properties that are different to those observed in the normal testis. Unfortunately, little more is known about its possible pathological and/or physiological roles at present. Some studies suggested that Dpl was anomalously turned over in neuron cells of Alzheimer disease (Ferrer *et al.,* 2004). The elucidation underlying the roles of Dpl and prion may not only serve as an insight into Dpl biology, but also provide an important window into prion diseases. In this study, we tried to elucidate the Dpl protein turn-over mechanisms involved, and discussed a possible relationship of the Dpl to ubiquitin proteasome system.

In order to determine if proteasomes play a role in the degradation some of Dpl, we examined the effect of proteasome inhibitors on the protein level of Dpl in HEK293 cells transfected with *PRND* cDNA encoding the full length Dpl. The transfected HEK293 cells were treated for 24 hr with 50 μM of the peptide aldehyde ALLN (Figure 1A) or with 10 μM of lactacystin (data not shown), and their Dpl protein content was analyzed in western blots with anti human-Dpl antibody. These inhibitors induced a large increase in the total amount of Dpl protein. To further characterize and study the intracellular sites of the accumulated Dpl by the proteasome inhibitors, we used immunofluorescent microscopy in permeabilized cells. As shown in Figure 1B, immunohistochemistry studies confirmed the aberrant Dpl expression in the *PRND* transfected cells that, similarly to the western blot analysis, HEK293 cells treated with ALLN presented a higher diffuse cytoplasmic protein content compared to the non treated cells. The cytoplasmic localization was also confirmed with fluorescent microscopy, following transfection of the HEK293 cells with the FL-Dpl-GFP expression plasmid

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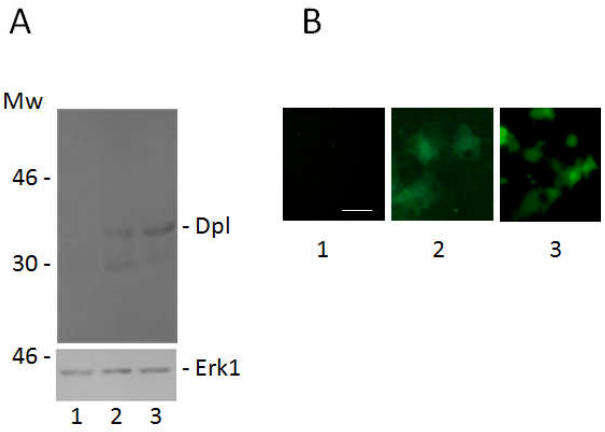

Fig. 1. Dpl accumulates in the cytoplasm of ALLN-treated cells. HEK293-Dpl cells transiently transfected with full length Dpl (PRND) were processed for Dpl immunoblot and immunofluorescence detection with anti-human Dpl antibody (Santa Cruz). The PRND-transfected cells were either untreated or treated with 12.5, 25 µM ALLN for 24 hr before lysis and analyzed on western blot (A), or immunostaining (B). Western blot with anti-Erk1antibody were also shown in Figure 1A lower panel, as equal levels of protein loading. Molecular markers (Mw) are indicated in the left in Kd. Bars (B), 10 micrometer.

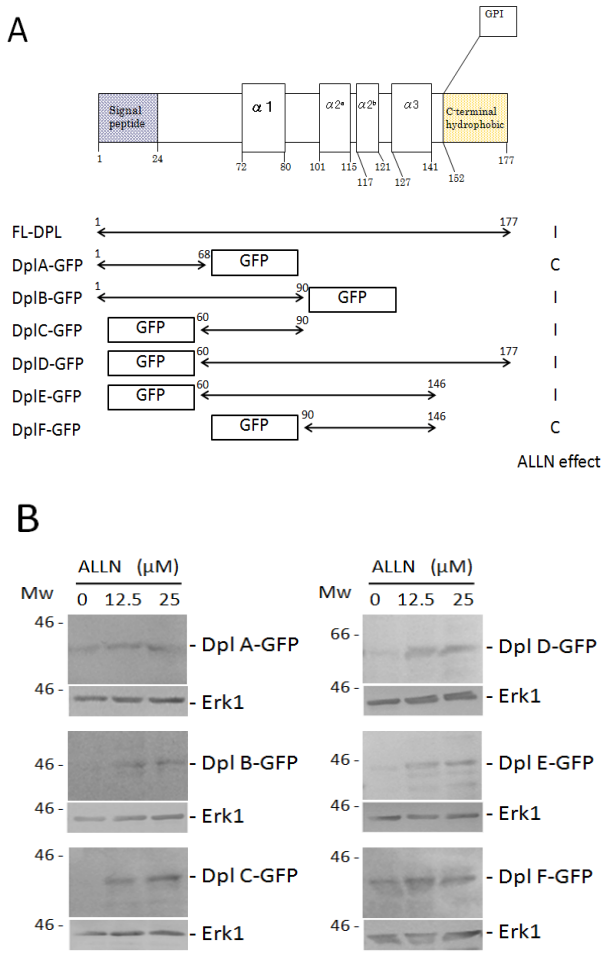

Fig. 2 A and B.. Schematic representation of full length (FL) Dpl and deletion mutant proteins used in panel B. The corresponding *PRND* sequence to the indicated portion of Dpl proteins, (DplA-GFP and DplB-GFP) or (DplC-GFP, DplD-GFP, DplE-GFP and DplF-GFP) were inserted into either pcDNA3. 1CT-GFP or pcDNA3.1NT-GFP plasmid, respectively. Constant (C) or increased (I) protein levels affected by ALLN treatment are indicated in the right. B. These plasmids were introduced into HEK293 cells together with a constant amount of PSV-GAL, followed by incubation for 48 hr. The amount of GFP protein expression was then measured by western blot and normalized as to expression of ERK1 (shown in panel B) and beta-galactosidase activity (not shown). HEK293 cells transiently expressing Dpl-deletion mutants fused to GFP (as in panel A) were lysed after treatment with ALLN (12.5 and 25 µM, 24 hr), and analyzed in western blot developed with anti-GFP antibody. Western blots with anti-Erk1antibody were also shown in each lower panel, as equal levels of protein loading.

(data not shown). In those cells, the Dpl accumulation was a time dependent and prominent after 20 hr treatment with ALLN in a range of 5-50 µM concentration (data not shown). Accordingly, we concluded that the ubiquitin proteasomal system appears to regulate the cytosolic Dpl protein level.

We next tried to map the effector domain for the ALLN treatment by testing various GFP-tagged deletion mutant proteins of Dpl, whether the domain was sensitive to the ALLN treatment. It should be noted that the full-length *PRND* cDNA clone was truncated as schematically depicted in Figure 2A and cloned into 2 kinds of GFP expression vectors. Transfected HEK293 cells with these GFP-tagged Dpl-mutant expression plasmids were then treated with 12.5 and 25 µM ALLN for 20 hr, and the cell lysates were used in subsequent western analyses with both anti-GFP and anti-Erk1 antibodies. Both of the Dpl A-GFP and the Dpl F-GFP proteins were certainly expressed and found by western blots even in the absence of ALLN, suggesting that these protein sites were not the target of ALLN treatment. In contrast to the results of them, the other domains of Dpl fused to GFP showed greatly increased amount of protein levels when treated with ALLN (Figure 2B). These analyses suggested that amino terminal regions surrounding the alpha-helix domain 1 (α1) was the right target site for responding to the ALLN.

That proteasome inhibitors increased the amount of Dpl suggested that proteasomes may be involved in the metabolism of this protein. And the α1 domain of Dpl may be involved in the mechanisms for protein degradation. To ascertain whether Dpl (especially α1 domain) interacted with ubiquitinated protein, HEK293 cells were transfected with Dpl C-GFP followed by ALLN treatment. Immunoprecipitated Dpl α1 domain using anti-GFP antibody showed the ladder bands indicating prominent anti-ubiquitin immunoreactive protein bound to Dpl α1 domain in the cells (data not shown). That ladder was confirmed by its presence in cells transiently expressing full length Dpl but not in mock transfected cells (data not shown). To study the possibility that the ladder contains ubiquitinated species, we again performed serial immunoprecipitation-western blot analysis (Figure 3 A). Following 20 hr incubation with ALLN, cells were lysed, and immunoprecipitates with anti-GFP antibody were then analyzed by western blot with anti-ubiquitin antibody. As shown in Figure 3A, several bands were seen in the ladder that reacted with both antibodies. Very faint ladder was detected in either non transfected HEK293 cells (data not shown), or in cells untreated with ALLN (Figure 3A, lane 1). Similar results were obtained in the experiments using Huh7 hepatoma cells instead of HEK293 cells (data not shown). Strikingly, a band of approximately 70 Kd (indicated by asterisk (*) in Figure 3A) seemed prominent in the ladder of those experiments. Because, the mobility of this band corresponds to the heat shock protein (HSP) 70, which is related to ubiquitin proteasome sysem, we speculated it as to HSP70. We then examined the biochemical in vivo interactions between Dpl and HSP70 (Figure 3B). Soluble cell lysates as used in Figure 3A were again prepared for immunoprecipitation

by using anti-GFP antibody, followed by western blotting using anti-HSP70 antibody. As shown in Figure 3B, GFP,

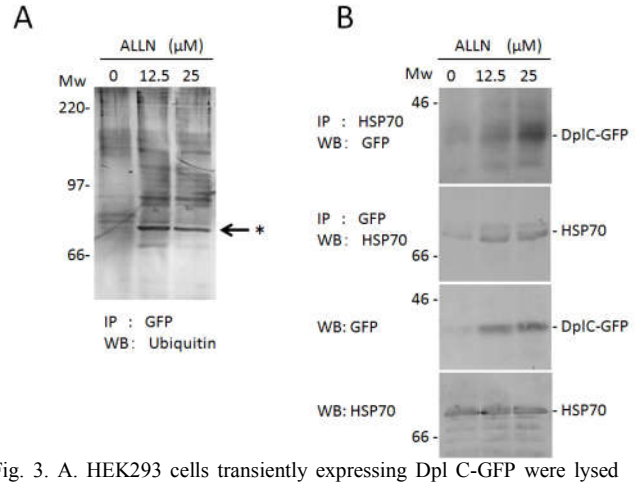

Fig. 3. A. HEK293 cells transiently expressing Dpl C-GFP were lysed after treatment with ALLN (12.5 and 25 μM, 24 hr), and the immunoprecipitates with anti-GFP antibody were then analyzed in western blot developed with anti-ubiquitin antibody (Santa Cruz). An asterisk arrowhead indicates the apparent 70 Kd band. B. Coimmunoprecipitaion of Dpl with HSP70. HEK293 cell lysates containing expressed Dpl C-GFP were immuneprecipitated by anti-GFP or anti-HSP70 antibodies (Santa Cruz), followed by SDS-PAGE and immunoblot analysis with anti-HSP70 or anti-GFP antibodies, respectively. Molecular markers (Mw) are indicated in the left in Kd.

but not untreated control, was coprecipitated with HSP70. Reciprocally, HSP70 was coprecipitated with HSP70 (Figure 3B), but not with the mock antibody (data not shown). These data clearly suggested that the alpha1 helix domain within Dpl protein could interact with HSP70 in the cells. We assumed that the HSP70 in the ladder that reacted with the ubiquitin antibody consisted Dpl-ubiquitinated proteins-HSP70 conjugates.

In the study presented here, we observed a dramatic accumulation of Dpl in cytoplasm of cells treated with a proteasomes inhibitor ALLN. A similar observation for prion had previously been reported (Yedidia *et al.,* 2001). Accordingly, we surmised that some Dpl protein may indeed be subjected to ubiquitin proteasome degradation system. To our knowledge, this is the first demonstration of Dpl modified by ubiquitination. It has been reported that the carboxyl terminus of HSP70-interacting protein (CHIP) plays a critical role in quality control of cellular proteins and stress recovery systems in most cell types (Qian *et al.,* 2006). CHIP functions as both a co-chaperone and an E3 ubiquitin ligase and serves as a molecular link between cellular protein folding and degradation. CHIP also mediates ubiquitin attachment to the chaperone substrate and stimulates the degradation of chaperone substrates (Meacham *et al.,* 2001).

Thus, regulating the levels and activity of HSP70 and CHIP may be candidates for Dpl degradation. More studies need to be undertaken to elucidate the precise molecular mechanisms.

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