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Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins

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In response to inflammatory stimulation, dendritic cells (DCs) have a remarkable pattern of differentiation (maturation) that exhibits specific mechanisms to control antigen processing and presentation. One of these mechanisms is the sorting of polyubiquitinated proteins in large cytosolic aggregates called dendritic cell aggresome-like induced structures (DALIS). DALIS formation and maintenance are tightly linked to protein synthesis. Here, we took advantage of an antibody recognizing the antibiotic puromycin to follow the fate of improperly translated proteins, also called defective ribosomal products (DRiPs). We demonstrate that DRiPs are rapidly stored and protected from degradation in DALIS. In addition, we show that DALIS contain the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme $E2_{25K}$, and the COOH terminus of Hsp70-interacting protein ubiquitin ligase. The accumulation of these enzymes in the central area of DALIS defines specific functional sites where initial DRiP incorporation and ubiquitination occur. Therefore, DCs are able to regulate DRiP degradation in response to pathogen-associated motifs, a capacity likely to be important for their immune functions.

Introduction

Dendritic cells (DCs) are regulators of the immune response, and control their antigen-processing activities in response to inflammatory stimuli (e.g., lipopolysaccharide [LPS]; Banchereau and Steinman, 1998). Among antigen-presenting cells, DCs are the most efficient at initiating antigen-specific responses, inducing differentiation of both naive CD4+ and CD8+ T cells (Banchereau and Steinman, 1998). In their immature state, DCs reside in peripheral tissues where they detect and capture incoming pathogens. DCs then begin a maturation process characterized by dramatic phenotypic and functional changes (Mellman and Steinman, 2001). Previously, we have shown that upon DC activation, large polyubiquitinated protein aggregates, named dendritic cell aggresome-like induced structures (DALIS), are formed (Lelouard et al., 2002). In contrast to classical aggresomes (Kopito, 2000), DALIS are neither localized in the pericentriolar area nor caged with vimentin (Heath et al., 2001). Moreover, DALIS form transiently, being detectable as soon as 4 h after DC activation and disappearing 24–36 h later (Lelouard et al., 2002). Interestingly, DALIS formation and maintenance requires continuous protein synthesis, suggesting that most of the molecular material incorporated in the aggregates is likely to be made of ubiquitinated newly synthesized proteins. Importantly, a large proportion of newly synthesized proteins (up to 30%) is known to be ubiquitinated and degraded by the proteasome shortly after synthesis (Wheatley et al., 1980; Schubert et al., 2000). Most of these substrates, termed defective ribosomal products (DRiPs), are not functional due to errors in the process of protein synthesis, such as misincorporation of amino acids and premature termination or deletion of residues, as well as post-translational mistakes occurring during folding, oligomer assembly, or intracellular triage. Recently, DRiPs have been proposed to be a major source of self or viral antigenic peptides for MHC class I restricted presentation (Reits

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Abbreviations used in this paper: CHIP, COOH terminus of Hsp70-interacting protein; DALIS, dendritic cell aggresome-like induced structures; DC, dendritic cell; DIC, differential interference contrast; DRiP, defective ribosomal product; LPS, lipopolysaccharide.

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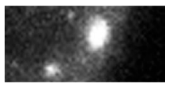
et al., 2000; Schubert et al., 2000; Khan et al., 2001; Yewdell and Bennink, 2001; Yewdell et al., 2001; Princiotta et al., 2003). Regulation of DRiP degradation in antigen-presenting cells could therefore affect the processing of many antigens, and thereby MHC class I restricted presentation (Schwab et al., 2003; Yin et al., 2003). Thus, we investigated the contribution of improperly translated proteins to DALIS formation during DC activation. The antibiotic puromycin was used to follow DRiPs both biochemically and visually during DC maturation. Puromycin is mistakenly inserted during protein synthesis by the ribosome in place of normal amino acids, resulting in truncated nascent polypeptide chains containing the drug at their COOH termini (Vazquez, 1974). This truncation creates a pool of aberrantly folded newly synthesized proteins rapidly degraded by the proteasome (Goldberg, 1972; Etlinger and Goldberg, 1980) and equivalent at least in part to natural DRiPs. Here, we report that puromycin-labeled DRiPs are targeted to DALIS <5 min after their synthesis. We also show that DALIS serve as specific zones for DRiP ubiquitination and storage over long periods, their center being particularly important in DRiP integration and ubiquitination. Therefore, DCs are able to regulate DRiP degradation through DALIS formation, a capacity likely to be important for their immune functions.

Results

Puromycin-labeled proteins are targeted to DALIS

DALIS formation and maintenance are abrogated by protein synthesis inhibitors, whereas drugs promoting abnormal protein synthesis, such as canavanin or puromycin, act as DALIS enhancers (Table I; Lelouard et al., 2002). Puromycin is an optimal tool to induce improper translation of proteins and establish the fate of this important population of antigens during DC activation. After incubation of LPS-treated mouse bone marrow-derived DCs (Inaba et al., 1992) with limited concentrations of puromycin for 30 min, we used a pAb able to detect puromycin-labeled proteins (Zhang et al., 1997) to perform immunofluorescence confocal microscopy (Fig. 1 A). Puromycin-labeled proteins were strongly enriched in DALIS, as demonstrated by their colocalization with polyubiquitinated proteins (Ub-proteins) in detergent-extracted DC. The puromycin found in the aggregates is clearly associated with newly synthesized proteins because puromycin detection in DALIS was eliminated by a cotreatment with the translation inhibitor cycloheximide (Fig. 1 B, CHX). Additional control experiments showed that puromycin treatment does not induce DC maturation or DALIS formation, and that puromycin-labeled proteins incorporated into DALIS are resistant to salt extraction (Fig. 1 B). Immunodetection of puromycin-labeled proteins in DCs, incubated with different concentrations of antibiotic before DALIS enrichment (Lelouard et al., 2002), indicated that 1 μg/ml is the optimal concentration allowing biochemical detection of puromycin-labeled proteins with minimal disturbance of cellular functions (Fig. 1 C). DC maturation considerably increased the quantity of high mol wt puromycin-labeled proteins (50–250 kD) detected in the DALIS-enriched fractions (Fig. 1 C, smear). These results suggest that the puromycin-labeled proteins, as already described (Etlinger and Goldberg, 1980), could be degraded like bona fide DRiPs in

Table I. **Effect on DALIS maintenance of different drugs altering protein synthesis**

	Concentration	Known effect on protein synthesis	Effect on DALIS
Control			
α-Amanitin	10 μg/ml	mRNA synthesis inhibition	
T-2 toxin	10 μg/ml	First peptidyl transfer (Met-AA_1) inhibition	
Anisomycin	10 μg/ml	Peptidyl transfer inhibition	
Cycloheximide	25 μM	Ribosomal translocation inhibition	
Emetine	25 μM	Ribosomal translocation inhibition	
Canavanin	15 mM	Arginine analogue giving rise to newly synthesized abnormal proteins	
Puromycin	5 μg/ml	Incorporation to the growing end of the polypeptide chain and translation premature termination giving rise to incomplete proteins (DRiPs)	

Drugs affecting either transcription or translation were added for 1 h to maturing DCs (8 h LPS) before DALIS observation by confocal microscopy.

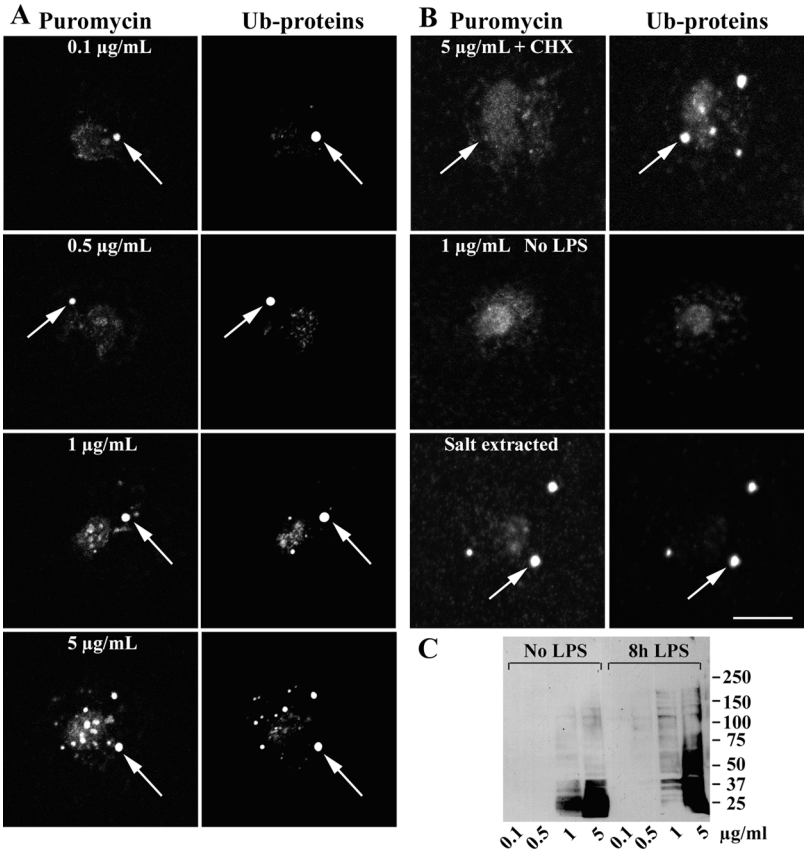


Figure 1. **Puromycin-containing proteins are targeted to DALIS during DC maturation.** (A) Maturing DCs (8 h LPS) were treated with 0.1–5.0 µg/ml of puromycin for 30 min before 1% Triton X-100 extraction and fixation. DCs were stained for puromycin (left) and ubiquitinated proteins (Ub-proteins, right), and were visualized by confocal microscopy. Puromycin is detected in DALIS (arrows). Note that the highest dose of puromycin (5 µg/ml) induced de novo formation of aggregates. (B) Top: co-treatment with 100 µM cycloheximide (CHX) abolishes puromycin incorporation in DALIS (arrow), which is solely due to newly synthesized puromycin-containing proteins. Middle: in immature DCs, no DALIS formation is observed, neither is accumulation of puromycin-containing proteins. Bottom: puro-proteins incorporated into DALIS resist sequential biochemical extraction with 1% Triton X-100, 100 µg/ml Dnase I, and 2 M NaCl. (C) Immature (No LPS) or maturing DCs (8 h LPS) were treated with 0.1–5.0 µg/ml puromycin for 30 min. DCs were submitted to biochemical extraction, and puromycin-containing proteins present in the final insoluble pellet were analyzed by immunoblot. Bar, 10 µm.

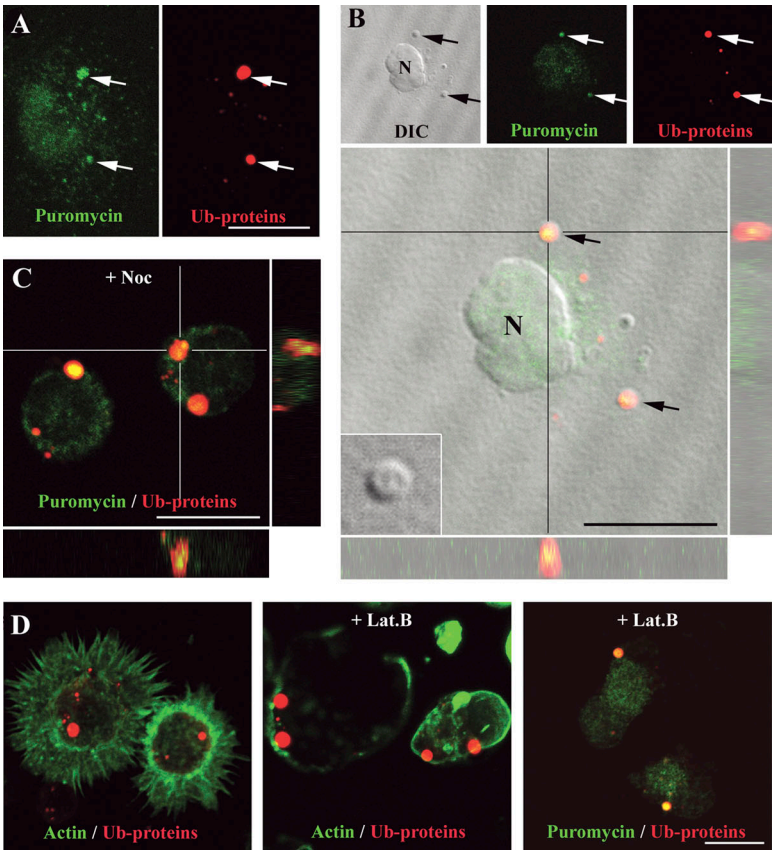


Figure 2. **Puromycin-labeled proteins are rapidly incorporated in the central area of DALIS by a mechanism independent of microtubules or actin filaments.** (A) Puromycin-labeled protein (green) incorporation into DALIS (red, arrows) is visualized as soon as 5 min after puromycin was added. (B) Maturing DCs were treated with 0.5 µg/ml puromycin for 15 min. After extraction with 1% Triton X-100, nucleus (N) and DALIS (arrows) were visualized by DIC or by immunofluorescence. Puro-protein (green) incorporation takes place right in the center of DALIS labeled by Ub-proteins (red), as illustrated by the yellow overlap in the combined xy, xz, and yz view. The inset displays a higher magnification view of a DALIS by DIC. (C) Disruption of microtubules with nocodazole before the puromycin pulse had no effect on puro-protein (green) incorporation in DALIS or on interaction between DALIS (red), as illustrated by the combined xy, xz, and yz view. (D) Disruption of actin network (stained by phalloidin in green, control on the left) with latrunculin B (Lat B) for 10 min before a 30-min puromycin pulse neither disrupted DALIS (Ub-proteins, red, center) nor prevented the incorporation of puro-proteins (green, right). Bars, 10 µm for all panels.

immature DCs, and indicate that part of these molecules are efficiently targeted to DALIS during DC maturation.

Puromycin-labeled proteins are rapidly incorporated in the central area of DALIS by a mechanism independent of microtubules or actin

We investigated the conditions of puromycin-tagged protein incorporation into DALIS during the early phase of DC maturation (8 h with LPS). This incorporation is extremely rapid; puromycin-labeled proteins were detected in the central zone of DALIS as soon as 5 min after adding the antibiotic to DCs (Fig. 2 A). This observation confirms the efficiency of misfolded protein targeting to DALIS and the potential implication of ribosomes in speeding up this process (Lelouard et al., 2002). Confocal Z-sectioning showed that puromycin-labeled proteins are incorporated at the center of the structure, and not simply by a more random aggregation in the periphery like classical aggresomes (Fig. 2 B; Kopito, 2000). Therefore, DALIS have a specific structural organization with a center extremely active in defective protein recruitment. Interestingly, differential interference contrast (DIC) microscopy revealed that DALIS are not fully homogenous spherical structures because contrasted areas can be detected either intruding in or protruding from their center (Fig. 2 B, enlarged frame). As expected, disrupting microtubules with nocodazole, which strongly impairs aggresome but not DALIS formation (Lelouard et al., 2002), had no effect on puromycin-labeled protein incorporation or DALIS organization as shown by Z-sectioning (Fig. 2 C). Treatment with latrunculin B, which destroys actin networks, did not interfere with puromycin-labeled protein accumulation in DALIS (Fig. 2 D). Therefore, these results indicate that DALIS are more complex than randomly associated macromolecular aggregates, and seem to possess architectural features able to incorporate newly improperly synthesized proteins actively and rapidly through their center.

Dynamics of DALIS and ubiquitin in live DCs

The rapid incorporation of ubiquitinated proteins in DALIS leads us to investigate this process in live DCs. We took advantage of a fusion of GFP with ubiquitin in COOH-terminal position (GFP-Ub), which was shown to behave as monomeric ubiquitin (Qian et al., 2002). In particular, GFP-Ub can be incorporated in ubiquitin polymers able to promote protein degradation. Therefore, GFP-Ub was expressed in live DCs using a retroviral system and was monitored by immunofluorescence microscopy (Fig. 3). GFP-Ub accumulation in

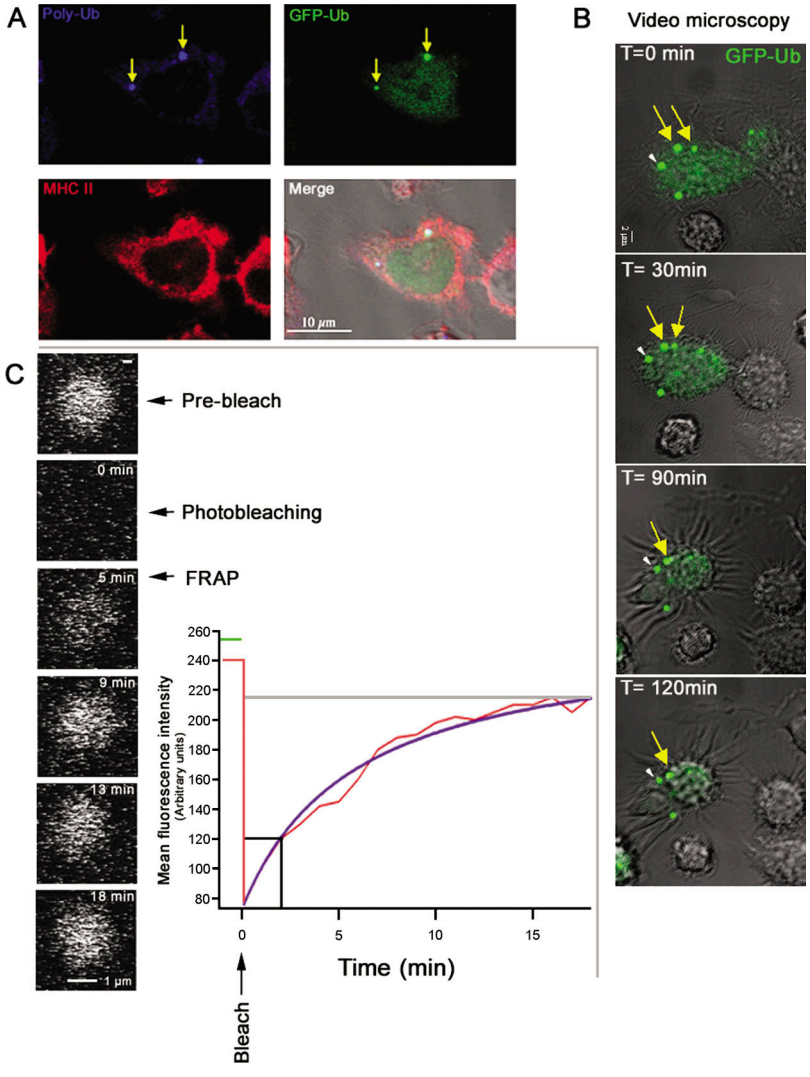


Figure 3. **DALIS are motile and constantly incorporate ubiquitin.** Using a retroviral infection system, bone marrow progenitors expressing GFP-tagged ubiquitin (GFP-Ub) were differentiated in DCs. (A) In MHC class II positive (red) maturing DCs, GFP-Ub (green) is targeted to DALIS (arrows) labeled with FK2 antibody (blue) as shown by confocal microcopy. (B) Video microscopy performed over 2 h demonstrates that in DCs visualized by DIC, GFP-Ub labeled DALIS (green, arrow) are motile and can fuse together. Large aggregates (arrowhead) are in general less motile. Bar, 2 μm. (C) FRAP was performed on DALIS. Representative images obtained before and after photobleaching are presented sequentially. The mean fluorescence intensity measurement (max = 240) plotted against time (real data in red and correlation curve in blue) is shown on the right. 50% fluorescence was recovered in 2 min, and 90% after 18 min.

DALIS was obvious from the intense fluorescence emanating from the polyubiquitinated aggregates in maturing DCs (Fig. 3 A). Video and time-lapse confocal microscopy performed on LPS-activated DCs expressing GFP-Ub revealed that DALIS are motile (~0.3–0.5 μm/min) and can interact together (Fig. 3 B and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200312073/DC1), suggesting that part of the aggregation process is mediated by fusion/interaction events. However, interactions were clearly preeminent in the smaller aggregates (<0.5 μm), indicating that DALIS are heterogeneous and that size itself has an important impact on DALIS behavior. The technique of FRAP was then used to monitor the dynamics of ubiquitin in DALIS at 8 h of maturation (Fig. 3 C and Video 1). 50% of the GFP-Ub fluorescence intensity was already recovered in 2 min, and almost full recovery was achieved 18 min after photobleaching. These observations emphasize the strong dynamics of ubiquitin and/or ubiquitinated protein recruitment in DALIS. Clearly, the FRAP experiments indicate that the fusion events observed by videomicroscopy are not necessarily required by large DALIS to acquire new ubiquitinated material. Furthermore, the relatively rapid speed of fluorescence recovery compared with the delay required for DALIS formation suggests that direct ubiquitination of proteins in DALIS could contribute (at least partially) to GFP-Ub incorporation in the aggregates.

Incorporation in DALIS delays protein degradation

Improperly translated protein incorporation into DALIS could also be followed kinetically by implementing puromycin pulse and chase experiments. To evaluate the clearing efficiency of the several washes performed before the chase, radioactive labeling was performed during either the 30-min

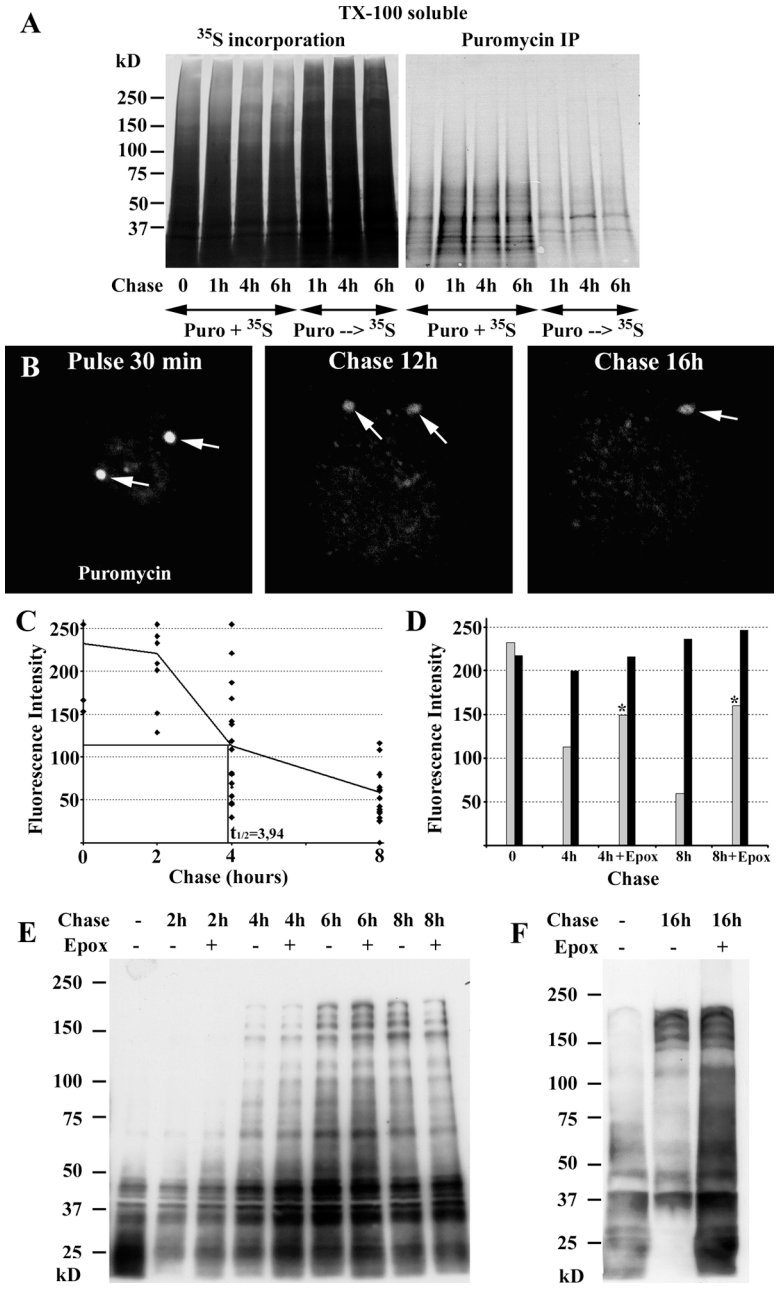


Figure 4. **DRiPs are ubiquitinated and stored in DALIS.** (A) maturing DCs (8 h LPS) were pulsed with 1 μg/ml puromycin together with ^{35}S-labeled Pro-Mix (puro + ^{35}S) for 30 min and chased for the indicated time. Alternatively, ^{35}S was added alone for 30 min at the indicated time during the puromycin chase (puro → ^{35}S). Puromycin clearance efficiency during the chase was monitored by autoradiography of 1% Triton X-100 soluble material (left) and by autoradiography of puromycin-labeled protein immunoprecipitation material (right). (B) Maturing DCs were pulsed with 1 μg/ml puromycin and chased for the indicated time. Puromycin-labeled proteins were visualized by confocal microscopy after detergent extraction. Even after 16 h of chase, puromycin-labeled proteins are detected in DALIS (arrows). (C) The maximal fluorescence intensity of puromycin-labeled proteins visualized by confocal microscopy was established for a statistically relevant number of DALIS ($15 < n < 20$) and was plotted against time of puromycin chase. (D) Proteasome inhibition (1 μM epoxomicin) effect on puromycin-labeled (gray bars) and polyubiquitinated (black bars) proteins stored in DALIS was estimated by the same fluorescence intensity measurement method (*, $P < 0.005$). (E) In the same experiment as above, puromycin-containing proteins were detected in DALIS-enriched fractions by immunoblot. The formation of high mol wt ladders, characteristic of polyubiquitination, could be observed with time, and this independently of proteasome inhibition (Epox). (F) After 16 h of chase, proteasome inhibition clearly prevented the loss of puromycin-labeled material from DALIS-enriched fraction, suggesting that DRiP degradation takes place at this late stage of maturation.

puromycin pulse or at different times after washing out the drug (Fig. 4 A). The detection of high mol wt proteins (normally lost in presence of puromycin; Fig. 4 A, left) and the extremely limited retrieval of immunoprecipitated [^{35}S] puromycin-tagged proteins when radioactivity was added after removing the antibiotic indicated that little contaminating puromycin was left or recycled during the chase. The residency time of improperly translated proteins in DALIS could therefore be evaluated during the early phase of DC maturation (8 h LPS) after a puromycin pulse of 30 min, followed by a chase of several hours. Puromycin-labeled proteins were still detected in DALIS by confocal microscopy after 16 h of chase, indicating that at least a fraction of these proteins is relatively long lived and resists degradation (Fig. 4 B), although a clear loss of fluorescent signal was observed. To estimate the time of puromycin-labeled protein residency in DALIS, the maximum intensity of fluorescence was measured in several large aggregates (>0.7 μm) and was correlated with the time of puromycin chase (Fig. 4 C). The half-time of residency was estimated to be 3.9 h, a period 23 times longer than the average half-life of DRiPs as monitored in fibroblasts (10 min; Princiotta et al., 2003). Interestingly, puromycin-tagged protein residency could be increased by the proteasome inhibitor epoxomicin, whereas the level of polyubiquitin present in DALIS remained relatively constant (Fig. 4 D). When the same samples were analyzed by immunoblotting of DALIS-enriched insoluble fractions, no loss of puromycin-labeled material during the chase could be detected (Fig. 4 E). On the contrary, over the 8-h time course a strong increase in high mol wt ladders, likely to correspond to ubiquitinated material (Schubert et al., 2000), could be observed. Moreover, proteasome inhibition had no effect on the total quantity of puromycin material recovered in the insoluble DALIS-enriched fraction during the 8 h of chase (Fig. 4 E). This suggests that the epoxomicin effect on the residency time in DALIS of puromycin-tagged protein (Fig. 4 D) was due to a relocalization or stabilization of these proteins in the aggregates rather than an inhibition of their degradation. However, proteasome inhibition during a longer period of chase (16 h) considerably increased the accumulation of all forms of puromycin-tagged molecules (Fig. 4 F), indicating that between 8 and 16 h of chase proteasomal degradation of puromycin-tagged proteins finally occurred.

Together, these results suggest that in maturing DCs, the major part of DRiPs can be polyubiquitinated and stabilized for periods (at least 8 h and up to 16 h) considerably longer than their described half-life (Princiotta et al., 2003). Interestingly, ubiquitination became obviously detectable 4 h after the pulse, a time at which the immunofluorescence data indicate 50% of the puromycin-labeled proteins have transited through large DALIS (0.7 μm < diameter < 2.1 μm). Therefore, DALIS may be susceptible to loose ubiquitinated material, still detectable by biochemistry in the insoluble fractions and protected from degradation for a long period. This may in part explain why DALIS growth is somehow limited to a maximum size. Therefore, DRiPs are probably ubiquitinated in DALIS and stored until proteasome-mediated degradation is initiated between 8 and 16 h later (16–24 h of maturation).

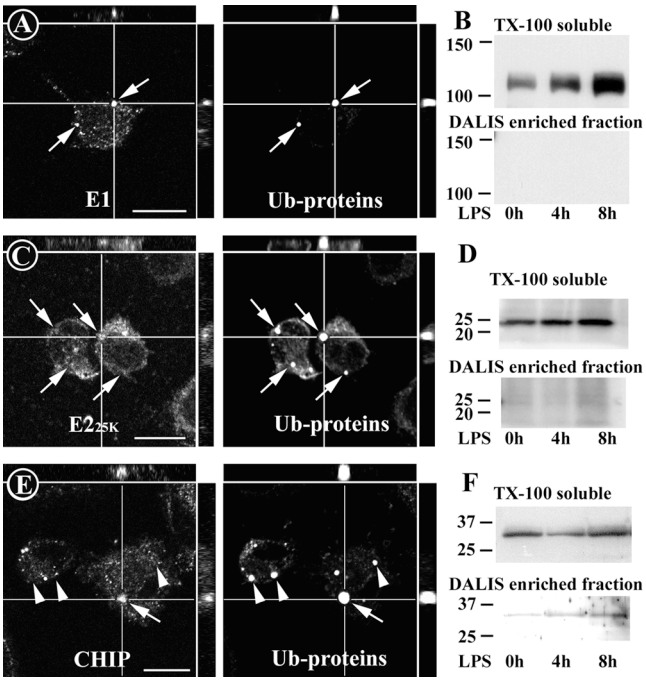


Figure 5. **Regulation and DALIS targeting of the ubiquitin-conjugation enzyme system during DC maturation.** (A) The E1 ubiquitin-activating enzyme (left) is present (arrow) in the central area of DALIS (Ub-proteins, right). (B) E1 expression strongly increased during the first 8 h of DC maturation without any change in its solubility, as monitored by immunoblot on soluble (top) and DALIS-enriched (bottom) fraction. (C) The ubiquitin-conjugating enzyme E2$_{25K}$ (left) is present (arrow) in DALIS (Ub-proteins, right). (D) E2$_{25K}$ expression increased during the first 8 h of DC maturation without any change in its solubility, as monitored by immunoblot on soluble (top) and DALIS-enriched (bottom) fraction. (E) The E3 ubiquitin ligase CHIP (left) is present (arrow) in the central area of some DALIS (Ub-proteins, right) and absent from others (arrowheads). (F) During DC maturation, expression of CHIP (as detected by immunoblot) increases in DALIS-enriched fraction (bottom), but not in Triton X-100 soluble fraction (top). Bars, 10 μm for all panels.

The ubiquitin-conjugating enzyme system is present in DALIS

The conversion with time of puromycin-labeled proteins into a ladder of high mol wt forms suggests that ubiquitination occurs in DALIS. Thus, we investigated whether the ubiquitin-activating enzyme E1 was present in DALIS. The detection by confocal microscopy of this enzyme in the aggregates (Fig. 5 A) confirmed our hypothesis and indicated that a ubiquitination-conjugating machinery is present in DALIS. E1 was found enriched at the center of the DALIS, confirming our previous finding on DALIS organization (Fig. 2 B). Interestingly, E1 expression was strongly increased during the first 8 h of maturation, but the enzyme could not be detected by immunoblotting in the insoluble DALIS-enriched fractions (Fig. 5 B), which indicates that at least a part of the DALIS functional machinery is sensitive to the biochemical extraction procedure.

In addition to the E1 ubiquitin-activating enzyme, we investigated by immunofluorescence the cellular localization of the ubiquitin-conjugating enzyme E2$_{25K}$, homologous to the yeast E2s UBC4/UBC5, which catalyses multi-ubiquitin

chain synthesis and mediates the selective degradation of short-lived and abnormal proteins (Chen and Pickart, 1990; Seufert and Jentsch, 1990). Like E1, $E2_{25K}$ was present in DALIS (Fig. 5 C), and its expression monitored by immunoblotting was increased during the first 8 h of maturation, but could not be detected in the insoluble DALIS-enriched fractions (Fig. 5 D).

Finally, in order to confirm that a complete ubiquitination machinery is present at the center of DALIS, we started to evaluate the cellular distribution of several E3 ubiquitin ligases. The E3 ubiquitin ligase, COOH terminus of Hsp70-interacting protein (CHIP), is a pivotal enzyme able to influence the refolding or the degradation of proteins both by interacting with heat shock molecules and mediating ubiquitination of misfolded substrates (Jiang et al., 2001; Meacham et al., 2001; Murata et al., 2001; Cyr et al., 2002). Therefore, CHIP was an interesting candidate E3 ubiquitin ligase to be tested in our system. CHIP was detected in DALIS by confocal microscopy (Fig. 5 D). Importantly, CHIP was not detected in all DALIS, suggesting that these structures might not all be equivalent, even in the same cells. As expected, CHIP was also enriched at the center of the DALIS, confirming the contribution of this zone in the active recruitment and the ubiquitination of defective protein. Conversely to E1 and $E2_{25K}$, CHIP was progressively enriched in insoluble DALIS fractions, whereas its expression in the soluble fraction remained constant (Fig. 5 E). Together, these results confirm the role of the central zone of DALIS in the ubiquitination of DRiPs, and indicate that the complete ubiquitin-conjugating enzyme system is regulated during DC maturation.

Discussion

DRiPs are considered as one of the most important sources of antigenic peptides presented by MHC class I in DCs and other cells (Reits et al., 2000; Schubert et al., 2000; Khan et al., 2001; Yewdell and Bennink, 2001; Princiotta et al., 2003; Yewdell et al., 2003). Using puromycin-labeled proteins as a model for DRiPs, we have unraveled their fate, which is likely to influence MHC class I presentation during DC maturation.

First, after their synthesis, DRiPs are rapidly targeted to the center of DALIS in maturing DCs. This rapid entry at a specific site implies that DALIS have a complex structural organization, probably involving specific scaffold proteins and enzymes likely to participate actively in improperly translated protein recruitment. Together with the fact that ribosomal proteins are found in close vicinity with DALIS (Lelouard et al., 2002), this observation may also indicate that the translation apparatus and its associated chaperone machinery could be directly involved in the process of selection and inclusion of DRiPs into DALIS.

Second, DRiP ubiquitination occurs at this central location. This was primarily suggested by the rapid replacement of the GFP-Ub in DALIS monitored by FRAP, and by the appearance of high mol wt puromycin-labeled proteins forming ladders in the DALIS-enriched fractions after chasing the antibiotic for relatively short times. The detection in DALIS of the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme $E2_{25K}$, and the E3 ubiquitin ligase CHIP, required respectively for all the steps of misfolded protein ubiquitination, confirms that DALIS can serve as ubiquitination areas for DRiPs. Their enrichment in the central area of DALIS also suggests that aberrant protein entry and ubiquitination at specific sites are well coordinated. Moreover, although CHIP, which is likely to bind the ubiquitination substrates, remains tightly associated with DALIS under stringent conditions, the E1 and $E2_{25K}$ enzymes are efficiently removed by the same treatment, indicating that soluble components are also part of the DALIS machinery. This situation clearly distinguishes DALIS from classical protein aggregates generally defined as simple storage units of insoluble molecules (Kopito, 2000). Importantly, the identified E1, E2, and E3 enzymes are all modulated during DC maturation; E1 and E2 being up-regulated while the insolubility of CHIP is increased, thus inferring that these enzymes have important function during DC maturation. The fact that ubiquitination occurs in DALIS also indicates that it is not a prerequisite for DRiPs targeting to DALIS, and may explain why other ubiquitinated proteasome substrates such as IkB-α are not incorporated in the aggregates (Lelouard et al., 2002).

Third, DRiP accumulation in DALIS considerably extends the half-life of these abnormal proteins by preventing proteasome-mediated degradation. The efficiency in delaying DRiP degradation is quite high, as judged by the inability of proteasome inhibition to increase the quantity of puromycin-labeled proteins detected by immunoblot in the first 8 h after puromycin incorporation (16 h after the activation of DCs). The estimated half-time of residency of puromycin-labeled proteins (4 h after the puromycin pulse) and the stability of polyubiquitin levels in DALIS during the first 8 h also implies the existence of an equilibrium between the number of DRiPs entering and exiting DALIS after ubiquitination. Moreover, it suggests that at this stage of DC maturation (8–16 h of maturation), part of the ubiquitinated DRiPs can be stored and protected from degradation, even after exiting large DALIS structures ($0.7\ \mu m <$ diameter $< 2.1\ \mu m$). In this condition, the stronger retention in DALIS of puromycin-labeled proteins observed after epoxomicin treatment could be due to an indirect effect of proteasome inhibition on many ubiquitin-based processes, rather than purely a block in the degradation of DRiPs stored in the aggregates.

Fourth, between 8 and 16 h of chase (16–24 h of DC maturation), DRiP degradation by the proteasome is clearly resumed.

DRiP fate during DC maturation has to be further integrated with the process of DALIS formation, maintenance, and disappearance. Videomicroscopy imaging using the GFP-Ub chimera indicates that DALIS are dynamic and can interact with each other (although in a heterogeneous manner). Therefore, DALIS formation could occur in several steps, first involving the assembly of "microaggregates," followed by the fusion of the smaller and apparently more dynamic aggregates, and ending with the stabilization of the larger structures storing at that point the bulk of the DRiP production. The integrity of the larger structures could be dependent on the activity of the translation machinery, as

demonstrated by the exquisite sensitivity of the aggregates to protein synthesis inhibitors. The translation machinery, in addition to producing DRiPs, could also provide some structural elements important to maintain the cohesion of large DALIS.

The efficient storage of large quantities of DRiPs during DC maturation is a puzzling phenomenon. The importance of DRiPs as an antigen source is such that a block/delay in their processing is likely to influence the MHC class I presentation in maturing DCs. The immunological consequences of this regulation remain to be fully evaluated. It has been recently proposed that DRiP processing and presentation is likely to be important for viral antigen presentation (Yewdell et al., 2003); the storage of important viral determinants by DCs would therefore appear to be a counterproductive defense strategy. However, new evidences suggest that viral antigen presentation by DCs is occurring mainly via cross-presentation (Lizée et al., 2003). This phenomenon is independent of direct viral protein synthesis by the DCs, and could be in fact favored by DALIS formation. Our work suggests that DALIS are organized structures performing coordinated biochemical functions, and that large protein aggregates might not be as amorphous as previously believed. Furthermore, the time frame of our observations implies that many important processes occurring in the first hours of DC activation have probably been overlooked.

Materials and methods

All chemicals were purchased from Sigma-Aldrich except epoxomicin, which was purchased from Affiniti Research Products.

Cell culture

7–8-wk-old male C57BL/6 mice were purchased from Charles River Laboratories. Bone marrow-derived DCs were cultured as described previously (Lelouard et al., 2002). mAbs TIB120, GK1.5, TIB 211, and B220 were obtained as hybridomas from the American Type Culture Collection (Gaithersberg, MD). Recombinant murine GM-CSF was produced as culture supernatant from J558L cells transfected with the murine GM-CSF cDNA (a gift of Dr. D. Gray, University of Edinburgh, Edinburgh, UK). Immature cell clusters were purified by unit gravity sedimentation on 6-ml 50% FCS columns. Maturation was induced using 100 ng/ml LPS.

Antibodies and immunocytochemistry

DCs were harvested and coated on 1% alcian blue–treated coverslips for 5 min at 37°C, permeabilized (or not) with 1% Triton X-100 in PBS for 5 min at 4°C, and fixed with 3% PFA in PBS for 10 min at RT. Rabbit pAb against puromycin was the gift of Peter Walter (University of California, San Francisco, CA). Rabbit pAb against CHIP was the gift of Cam Peterson (University of North Carolina, Chapel Hill, NC). mAb FK1, FK2, and rabbit pAb against E1 and $E2_{25K}$ were obtained from Affiniti Research Products. mAb against E1 was from Sigma-Aldrich. All Alexa® secondary antibodies were from Molecular Probes, Inc. Immunofluorescence and confocal microscopy (using microscope model LSM 510; Carl Zeiss MicroImaging, Inc.) were performed as described previously (Lelouard et al., 2002).

Pharmacological treatments

DCs were incubated, unless stated otherwise, with 0.5 or 1 μg/ml puromycin for 30 min. 1 μM epoxomicin was added for indicated times. 100 μM cycloheximide, 5 μM latrunculin B, or 10 μg/ml nocodazole were added 10 min before puromycin pulse.

Live imaging and FRAP

GFP-ubiquitin cDNA was the gift of J. Yewdell (National Institutes of Health, Bethesda, MD), and the LZRS retroviral vector was from G. Nolan (Stanford Universitty, Stanford, CA). Bone marrow progenitors were infected with LZRS recombinant retroviruses engineered to express GFP-Ub as described previously (Chow et al., 2002). After differentiation in DCs, GFP-expressing cells incubated for 8 h with LPS were used for live imaging. Videomicroscopy at 37°C in 5% CO_2 was performed with an inverted microscope (Leica) equipped with a PentaMAX™ CCD camera (Princeton Instruments). Images were collected every 2 min for 3 h and processed with MetaMorph® version 5 (Universal Imaging Corp.). FRAP was performed on a confocal microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) using an argon laser 488-nm ray with a power of 12.5 mW applied for 5 s to a selected spot containing DALIS (average of 1-μM radius). Fluorescence recovery was observed at 37°C by collecting 15 consecutive 0.5-μM slices every 2 min with a laser power of 1.25 mW. Images were analyzed and quantified with LSM Image Examiner software (Carl Zeiss MicroImaging, Inc.), MetaFluor version 5.0 (Universal Imaging Corp.), and Igor (Wavemetrics). No obvious loss of fluorescence was detected in control areas after FRAP on DALIS.

Selective enrichment of DALIS

DCs were submitted to a 30-min extraction with 1% Triton X-100 in 20 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, and 10 mM NaCl at 4°C before a 30-min DNase (100 μg/ml) treatment at 37°C in the same buffer. Nuclear material extraction was then performed for 30 min at 4°C with 2 M NaCl in 20 mM Tris, pH 7.4, and 1.5 mM $MgCl_2$. Remaining material was PBS washed and submitted to immunocytochemical and biochemical analysis.

Radiolabeling, immunoprecipitation, and immunoblots

10^6 DCs were pulse labeled with 1 μg/ml puromycin, and in some cases with 10 mCi/ml of [^{35}S]methionine Pro-Mix (APB) for 30 min, washed, and chased for various time at 37°C in RPMI 1640/5% FCS as described previously (Lelouard et al., 2002). Triton X-100 soluble or DALIS-enriched fractions were analyzed before or after immunoprecipitation with anti-puromycin antibody. Material separation onto 2–10% gradient SDS-PAGE gels and immunoblots was performed as described previously (Lelouard et al., 2002) and was revealed by chemiluminescent detection (Pierce Chemical Co.).

Online supplemental material

Time-lapse confocal microscopy (five slices of 0.5 μm every 2 min for 1 h) followed by a FRAP experiment performed on GFP-Ub expressing DCs after 8 h in LPS. Pictures were superimposed and assembled sequentially in time. Small DALIS fusion with larger aggregates can be observed as well as rapid FRAP. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200312073/DC1.

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