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Critical Role for Asparagine Endopeptidase in Endocytic Toll-like Receptor Signaling in Dendritic Cells

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SUMMARY

Intracellular Toll-like receptor 3 (TLR3), TLR7, and TLR9 localize in endosomes and recognize single-stranded RNA and nucleotides from viruses and bacteria. This interaction induces their conformational changes resulting in the production of proinflammatory cytokines and upregulation of cell surface molecules. TLR9 requires a proteolytic cleavage for its signaling. Here, we report that myeloid and plasmacytoid dendritic cells (DCs) deficient for the asparagine endopeptidase (AEP), a cysteine lysosomal protease, showed a decrease in the secretion of proinflammatory cytokines in response to TLR9 stimulation in vitro and in vivo. Upon stimulation, full-length TLR9 was cleaved into a 72 kDa fragment and this processing was strongly reduced in DCs lacking AEP. Processed TLR9 coeluted with the adaptor molecule MyD88 and AEP after size exclusion chromatography. When expressed in AEP-deficient DCs, the 72 kDa proteolytic fragment restored TLR9 signaling. Thus, our results identify an endocytic protease playing a critical role in TLR processing and signaling in DCs.

INTRODUCTION

Toll-like receptors (TLRs) recognize microbial products and play an essential role in innate and adaptive immunity. They belong to the type I transmembrane protein family that contains a leucine-rich repeat in an extra cellular loop and a Toll-interleukine-1 (IL-1) receptor (TIR) homology domain in the cytoplasmic tail (Bell et al., 2003). TLRs are divided in two families: TLR1, 2, 4, 5, 6, and 11, which sense the presence of proteins and lipids from bacteria and are expressed at the plasma membrane; and TLR3, 7-8, and 9, localized in endosomes (Kawai and Akira, 2006; Kim et al., 2008), which engage single-stranded and double-stranded RNA and unmethylated CpG DNA, respectively, from pathogens. In the absence of stimulation, TLR9 is retained in the endoplasmic reticulum (ER) together with another ER-resident protein, UNC93B (Brinkmann et al., 2007). Upon stimulation with CpG DNA, it relocates to the endo-lysosomal compartment, allowing the recruitment of the adaptor molecule, MyD88, and thereafter, signals. Recently it was demonstrated that in macrophages, after stimulation, TLR9 is cleaved into a C-terminal fragment and that this cleaved form alone is competent for signaling (Ewald et al., 2008). This is an important step indeed, because it is the activation of the MyD88 signaling pathway that leads to the production of proinflammatory cytokines and cell surface expression of costimulatory molecules in dendritic cells (DCs) (Hemmi and Akira, 2005; Kaisho and Akira, 2001). It has further been shown that interfering with the pH by blocking lysosomal acidification inhibits TLR7 and 9 signaling (Yi et al., 1998), suggesting a role for endocytic proteases in endosomal TLR stimulation.

Lysosomal proteases, which are highly dependent on the pH for their activities, can be classified into three major classes: aspartic, serine, and cysteine protease families. Asparagine endopeptidase (AEP) or mammalian legumain is an asparagine-specific cysteine protease that has been implicated in the MHC class II presentation pathway. AEP initiates the processing of tetanus toxin in human B cells, destroys an immuno-dominant peptide of myelin basic protein (MBP, 85-99), and performs the early steps of degradation of the invariant chain (Ii) in human B-EBV cells (Manoury et al., 1998, 2002, 2003). AEP is unrelated to the papain-like cysteine protease family such as cathepsins B (CatB) and L (CatL); it is grouped together with the caspases, separases, and some bacterial proteases in clan CD (Chen et al., 1997, 1998; Uhlmann et al., 2000). Unlike other lysosomal cysteine proteases, AEP is insensitive to leupeptin and cleaves on the carboxy-terminal sides of asparagine residues. AEP is synthesized as a 56 kDa precursor and targeted to the endocytic pathway. In the lysosomal compartments, N- and C-terminal propeptides are auto-cleaved to generate a 46 kDa mature form, which can be further processed into a 36 kDa fragment (Li et al., 2003). Acidic pH is a prerequisite for maturation of AEP, and therefore its greatest activity is found in lysosomal compartments. AEP is constitutively expressed in most cell types, especially in macrophages and DCs. Recent published findings have centered around cysteine proteases (in particular cathepsin K and cathepsin L) in endosomal TLR activation (Asagiri et al., 2008; Matsumoto et al., 2008; Park et al., 2008). However, no direct biochemical evidence in DCs for TLR9 cleavage by cathepsin L or cathepsin K was brought forward in

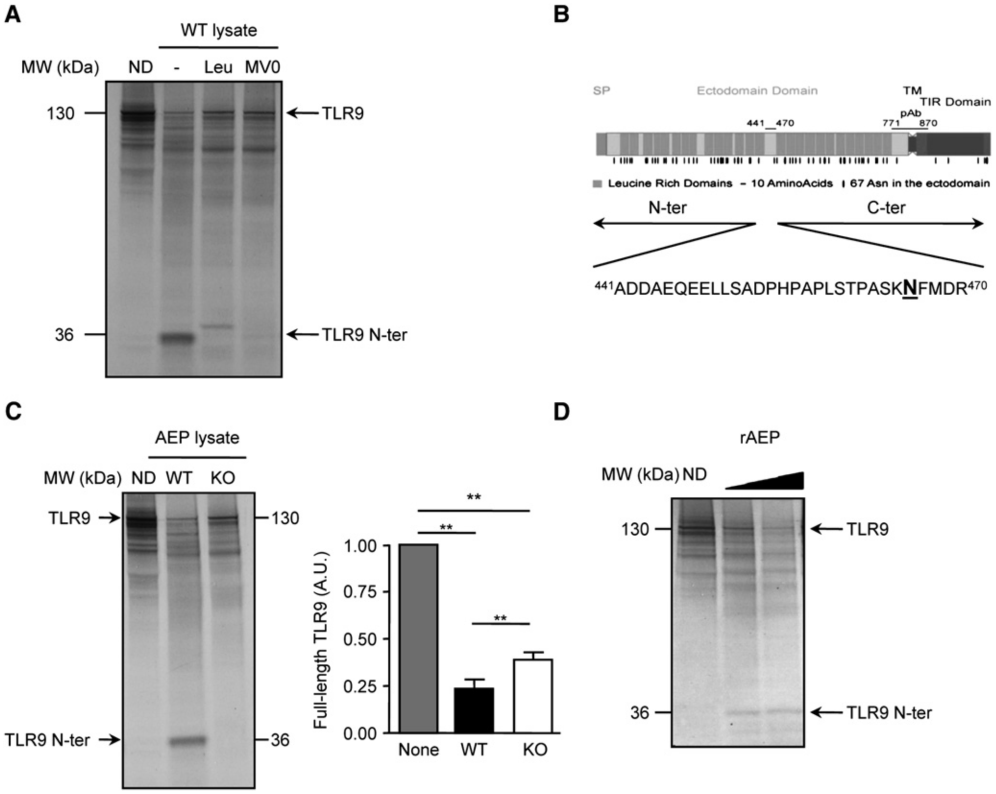

Figure 1. Digestion of ^{35}S-Labeled TLR9 with Purified AEP or Disrupted Lysates from WT or AEP-Deficient BMDCs

(A) *Tlr9* cDNA was transcribed and translated in vitro in the presence of rabbit reticulocyte and ^{35}S-Met. ^{35}S-Met-labeled TLR9 protein was digested or not with 20 μg of WT cell lysate in the absence or presence of the indicated inhibitors for 4 hr.

(B) Scheme of murine TLR9 protein (Uniprot accession number Q9EQU3). The 26 LRRs and recognition domain of polyclonal rabbit TLR9 (Santa Cruz) are represented as well as the putative Asn cleavage site, pI, and MW of fragments.

(C) Same reaction as (A) via WT (lane 2) or AEP-deficient (KO, lane 3) DC lysate (left). Graphs show mean ± SD (n = 3) via one-way ANOVA test. **$p < 0.01$ (right).

(D) Same digestion experiments with different doses of rAEP (7.5U, lane 2; 15U, lane 3). The reactions were performed in 50 mM citrate buffer, 0.1% CHAPS (pH 5.5) and separated on a 10% SDS-NuPAGE. ND, nondigested.

Representative data from three independent experiments.

those studies. With this in mind, we decided to investigate whether AEP was required for TLR9 response. Here, we showed that full-length TLR9 was a substrate for AEP both in vitro and in living cells. Blocking AEP activity by knocking down its gene decreased the appearance of TLR9 proteolytic fragments of 36 and 72 kDa in murine DCs, resulting in reduced TLR9 signaling after CpG stimulation in cells and in AEP-deficient ($Lgm^{-/-}$) mice. Our resulting findings suggest that AEP plays a major role in TLR9 signaling in DCs.

RESULTS

AEP Can Cleave TLR9 In Vitro

To analyze the processing of TLR9 in vitro, we incubated radiolabeled translated TLR9 with lysate of wild-type (WT) bone-marrow-derived DCs (BMDCs) as a source of proteases. The full-length TLR9 protein was digested to produce one major band that migrates around 36 kDa (Figure 1A), corresponding probably to the N-terminal part of TLR9 (TLR9 N-ter) (Figure 1B) previously described (Park et al., 2008). We then used various protease inhibitors to identify the enzymes involved in TLR9 degradation in vitro. In the presence of leupeptin (a broad inhibitor of cysteine proteases except AEP), full-length TLR9 was digested to generate a fragment different from the TLR9 N-ter migrating at a higher molecular weight (Figure 1A). In contrast, addition of MV026630, previously described to specifically inhibit AEP (Loak et al., 2003; Manoury et al., 2003), showed a clear inhibition of the production of the TLR9 N-ter product (Figure 1A). To investigate this further, we used lysate from WT and AEP-deficient BMDCs as a source of proteases. Proteases contained in both lysates were able to degrade full-length TLR9 (Figure 1C), but those expressed in AEP-deficient BMDC lysate digested TLR9 less efficiently than WT lysate. Longer exposure of the radioactive film showed that both lysates could cleave TLR9 into fragments (Figure S1A available online) among which two of them migrate at 72 and 36 kDa corresponding probably to the N- and C-terminal part of TLR9 fragments (Figure 1B). To test whether AEP could directly cleave TLR9, TLR9 was incubated with recombinant AEP, at different doses. Recombinant AEP was able to cleave TLR9 into fragments, one of them being the TLR9 N-ter (Figure 1D). As previously described (Ewald et al., 2008; Park et al., 2008), cathepsins K (CatK), L (CatL), and S (CatS)