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Proinflammatory effects of pancreatic elastase are mediated through TLR4 and NF-κB☆

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Abstract

Pancreatic elastase has been implicated in the pathophysiology of severe acute pancreatitis, characterized by systemic inflammatory response, distant organ failure, and high mortality. Here we show that pancreatic elastase activates transcription factors NF-κB, AP-1, and NFAT in human myeloid cells (U-937 and THP-1) in culture. Pancreatic elastase also induces TNF-α secretion and increased expression of CD11b in THP-1 cells which can be inhibited by neutralizing anti-Toll-like receptor 4 (TLR4) antibodies. NF-κB blocking agents (MG-132, PGA_1) prevented elastase-induced TNF-α secretion from THP-1 cells. Our results suggest that pancreatic elastase-induced proinflammatory effects are mediated by TLR4 and NF-κB in human myeloid cells.

Keywords: Acute pancreatitis; CD11b; Elastase; NF-κB; TLR4

Prematurely activated pancreatic enzymes may have a key role in the initiation and propagation of pancreatic damage during acute pancreatitis [1,2]. In addition to pancreatic injury, the pathophysiology of severe acute pancreatitis involves systemic inflammatory response often resulting in distant organ dysfunction and failure [3]. Activated inflammatory cells and substances secreted by them have been implicated in the propagation and amplification of systemic inflammatory response in acute pancreatitis. The initiating mechanisms remain obscure, however.

Recent studies suggest that pancreatic enzymes, such as elastase, have proinflammatory properties besides their digestive enzymatic activity. Pancreatic elastase activates nuclear factor-κB (NF-κB) and induces secretion of tumor necrosis factor-α (TNF-α) from mononuclear inflammatory cells in vitro and in experimental animals [4,5]. A Toll-like receptor 4 (TLR4), which has been shown to be critical to the effects of lipopolysaccharide, has also been implicated in heparan sulfate and elastase-induced inflammatory response and mortality in a mice model [6].

In this study, we have studied the effects of pancreatic elastase on cultured myeloid cells. We show that the proinflammatory effects of pancreatic elastase, such as TNF-α secretion and increased expression of CD11b, are mediated by TLR4, and TNF-α secretion also by NF-κB.

Materials and methods

Cells and materials

Human myeloid cell lines U-937 and THP-1 were obtained from ECACC (European Collection of Cell Cultures, CAMR, Salisbury,

☆ *Abbreviations:* AP-1, activating protein-1; EMSA, electrophoretic mobility shift assay; IκB, inhibitory-κB; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-κB; PGA_1, prostaglandin A_1; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.

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UK). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 mg/ml streptomycin and 100 U/ml penicillin (Gibco Europe, Paisley, UK). Porcine pancreatic elastase (#324723) and human recombinant tumor necrosis factor-α (TNF-α) (#654205) were purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). Lipopolysaccharide (LPS, *Escherichia coli* serotype 026:B6) was from Sigma Chemical (St. Louis, MO, USA). MG-132 was from Calbiochem and PGA_1 was from Cayman Chemicals (Ann Arbor, MI, USA). Other chemicals and reagents were from Sigma Chemical (St. Louis, MO, USA).

Experimental protocol

In the present study, pancreatic elastase concentration of 1 U/ml was used in all experiments. Based on preliminary studies, this concentration was found to be non-toxic while stimulating the cells. For experiments involving nuclear- and cytoplasmic-protein extractions, the U-937 and THP-1 cells (10^7) were resuspended in fresh media on 35 mm culture plates 12 h prior to experiments. After stimulation with elastase, TNF-α of LPS, the cells were collected, washed in ice-cold PBS-EDTA (2 mM) with protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride (AEBSF, Roche Molecular Biochemicals, Mannheim, Germany), and processed for nuclear- and cytoplasmic-protein extracts as described by Schreiber et al. [7]. Protein concentrations were determined by the method of Bradford [8]. Cell proliferation and viability was assessed by colorimetric XTT-assay (Roche Molecular Biochemicals, Mannheim, Germany).

Transcription factor assays

Electrophoretic mobility shift assay (EMSA). Aliquots of 7.5 μg of nuclear protein were mixed in 20 μl reactions containing 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DDT, Tween 20 0.2% (w/v), 30 mM KCl, and 0.05 μg/ml poly(dI–dC) and 0.05 g/ml poly-L-lysine according to manufacturer's instructions (Roche, Dig Gel Shift Kit, Mannheim, Germany). The oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, Promega, Madison, WI, USA) containing κB-binding motif was end labeled with digoxigenin-11-ddUTP according to manufacturer's instructions (Roche, Dig Gel Shift Kit). 0.04 ng/ml of the probe was added to the mixture and the binding reaction was allowed to proceed for 20 min at room temperature. DNA–protein complexes were resolved in a 5% non-denaturing polyacrylamide gel in a 0.5× TBE buffer at 100 V for 1 h. The gels were then transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 0.4 A. The membranes were then cross-linked with UV-light for 3 min. Thereafter, the membranes were blocked, washed, and incubated with anti-Dig-antibody and the protein–DNA complexes were visualized by CSPD chemiluminescence according to manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Transient transfections. U-937 cells were seeded from low-passage cultures at $5–6 \times 10^5$ cells/ml in RPMI-1640 w/o FBS or antibiotics. The cells were then transiently transfected with secreted alkaline phosphatase (SEAP) vectors (pNF-κB-SEAP, pAP1-SEAP, pNFAT-SEAP, and pHSE-SEAP) or the control vector (pTAL-SEAP) (BD Biosciences, Clontech) in 35 mm culture dishes using Fugene 6 transfection reagent according to manufacturer's instructions (Roche). The cells were co-transfected with pTAL-Luc vector to normalize transfection efficiencies. At 20 h post-transfection, the cells were washed, resuspended in fresh media, divided into 48-well plates, and stimulated. The activity of SEAP was assayed from culture fluid by using 4-methylumbelliferyl phosphate as substrate [9]. Fluorescence was analyzed by a fluorescence plate reader (Victor-2 1420 Multilabel Counter, Wallac, Finland) with excitation set at 360 nm and emission at 460 nm. Luciferase activity was assayed from cell homogenates by Luciferase Reporter Assay kit (BD Clontech). The results are expressed as relative SEAP activity over control and normalized against luciferase activity.

TransFactor assay. An enzyme linked immunosorbent assay (ELISA)-based transcription factor assay was used (BD Mercury TransFactor Kit, BD Biosciences, Palo Alto, CA, USA) to quantitatively measure transcription factor activation. In brief, aliquots of nuclear protein extracts were added onto a 96-well plate coated with a specific DNA-binding sequence for each transcription factor studied (NF-κB, AP-1). Primary (p65, c-Fos) antibodies against transcription factor subunits and secondary antibodies were then used to detect bound transcription factors. Finally, the absorbances were analyzed by a microplate reader.

Western blot analysis. Equal amounts of nuclear-protein extracts (5 μg) were diluted in Laemmli sample buffer with 5% mercaptoethanol. After boiling, the samples were resolved in 10% polyacrylamide gels in Tris–glycine–SDS buffer. The gels were transferred into nitrocellulose membranes, blocked in 5% non-fat dried milk in phosphate-buffered saline (PBS), pH 7.5, containing 0.1% (vol/vol) Tween 20 (PBST-milk). Blots were then incubated with polyclonal rabbit anti-p65 antibody (sc-372) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 1:1000 (vol/vol) dilution in PBST-milk at 4 °C overnight. The membranes were washed in PBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG at 1:5000 (vol/vol) dilution in PBST-milk for 1 h. After washing, the protein bands in the membranes were visualized by enhanced chemiluminescence (Supersignal West Pico, Pierce Biotechnology, Rockford, IL, USA).

TNF-α assay. TNF-α secretion from THP-1 cells was measured from culture fluid by using a commercial ELISA kit (Quantikine HS, RD Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The absorbances were read by a microplate reader and concentrations were calculated according to standard curve.

TLR-4 and NF-κB blocking. THP-1 cells were preincubated in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (1×10^6 cells/ml, 37 °C, 5% CO_2) with mouse anti-human TLR4 antibody 10 mg/ml (HTA125, MCA2061XZ, Serotec, Oxford, UK) or with isotype specific control mouse IgG_{2a} (OX34, MCA929XZ, Serotec, Oxford, UK) for 1 h before adding pancreatic elastase (1 U/ml). For NF-κB inhibition, either MG-132 (25 μM, Calbiochem, San Diego, CA) or PGA1 (100 mM, Cayman Chemicals, Ann Arbor, MI, USA) was used.

Flow cytometry. THP-1 cells (1×10^6 cells/ml) were either left unstimulated or stimulated with elastase (1 U/ml) for 72 h at 37 °C in 5% CO_2. TLR4 inhibition was performed as described before. After the stimulation, the cells were washed twice with PBS and resuspended in PBS with FITC-conjugated mouse monoclonal anti-CD11b or with FITC-conjugated isotype matched control mouse IgG_1, (sc-1186 FITC, sc-2855 FITC, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at 4 °C. Thereafter, the cells were washed twice in PBS and analyzed on a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) set to detect fluorescence, forward scatter, and side scatter. Non-specific fluorescence was determined by isotype matched mouse IgG as control antibody.

Analysis of data

The results reported in this communication represent the mean ± SEM obtained from 3 or more separate experiments. In all figures, vertical bars denote SEM values. Statistical evaluation of data was accomplished by analysis of variance (ANOVA) and p values of less than 0.05 were considered significant.

Results

Pancreatic elastase activates several transcription factors

Pancreatic elastase induced activation of NF-κB, AP-1, and NFAT transcription factors tested at 3 h (Fig. 1A). Elastase-induced NF-κB activation was not as rapid as the activation induced by TNF-α or LPS (30 min) but was clearly present at 3 h (Fig. 1B). Pancreatic elastase was non-toxic at the concentration used (1 U/ml). The viability and cell proliferation index (XTT-test) of elastase-stimulated U-937 and THP-1 cells did not differ from controls at 48 h (data not shown).

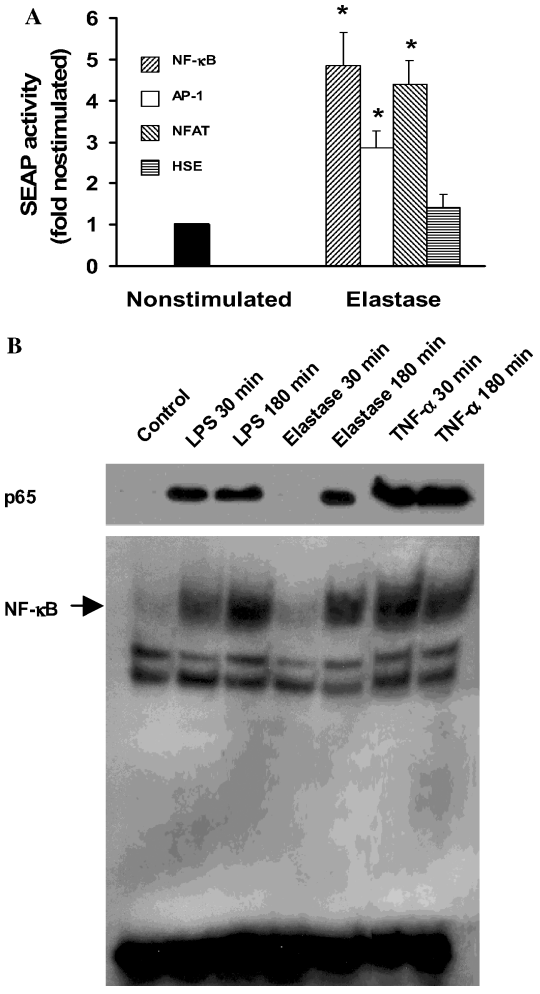

Fig. 1. Effect of pancreatic elastase on transcription factor activation. (A) U-937 cells were transiently transfected with vectors (pNF-κB-SEAP, pAP1-SEAP, pNFAT-SEAP, and pHSE-SEAP). The cells were incubated with pancreatic elastase (1 U/ml) for 3 h and secreted alkaline phosphatase (SEAP) activity was assayed from the culture medium as described in Materials and methods. SEAP activities were normalized against luciferase activity (transfection efficiency) and transcription factor activation expressed as fold non-stimulated control. *Denotes $p < 0.05$, ANOVA. (B) U-937 cells were incubated for 30 and 180 min with pancreatic elastase (1 U/ml), LPS (10 μg/ml), and TNF-α (10 ng/ml). Nuclear extracts were then prepared and assayed for NF-κB (EMSA) and p65 (Western blot).

Pancreatic elastase-induced TNF-α secretion is NF-κB dependent

Elastase-induced NF-κB activation was inhibited by MG-132 (26S proteasome inhibitor) and by prostaglandin A1 (PGA_1, inhibitory κB-kinase inhibitor). MG-132 and PGA_1 effectively inhibited elastase-induced NF-κB activation (p65 nuclear translocation). They also induced AP-1 activation by themselves (c-Fos nuclear translocation) and had no effect on elastase-induced AP-1 activation (Fig. 2A). Both of the inhibitors effectively prevented elastase-induced TNF-α secretion from THP-1 cells (Fig. 2B), suggesting that elastase-induced TNF-α secretion is mediated by NF-κB activation.

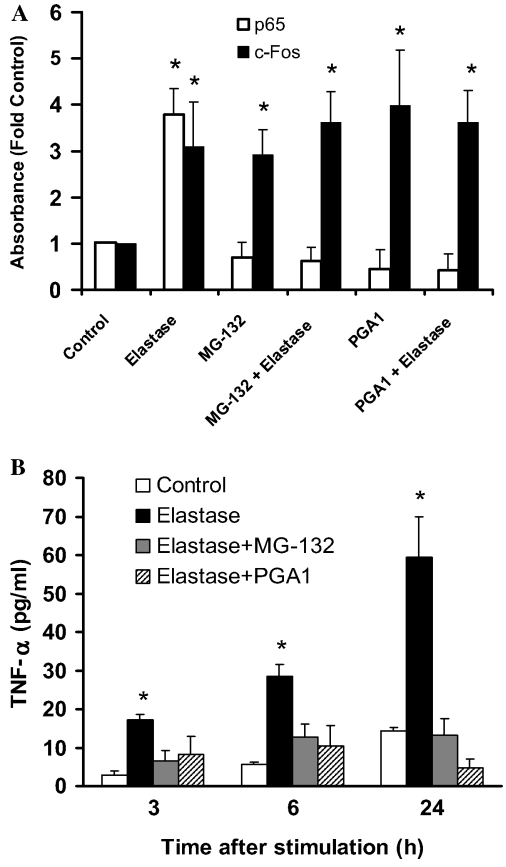

Fig. 2. The effect of NF-κB inhibitors on elastase-induced transcription factor activation and TNF-α secretion. (A) Transcription factor activation. THP-1 cells were preincubated (1 h) with either MG-132 (25 μM) or PGA_1 (100 μM) before stimulation with pancreatic elastase (1 U/ml) for 3 h. Nuclear protein extracts were prepared and incubated in wells (96-well plate) coated with NF-κB or AP-1 DNA consensus sequence. Primary antibodies against transcription factor subunits (p65 for NF-κB and c-Fos for AP-1) were used and then detected by HRP-conjugated secondary antibodies and substrate. The results are expressed as fold non-treated and non-stimulated control. (B) TNF-α secretion in response to elastase stimulation. THP-1 cells were preincubated for 1 h with either MG-132 (25 μM) or PGA_1 (100 μM) before adding pancreatic elastase (1 U/ml). TNF-α concentration was measured from the culture media until 24 h. *Denotes $p < 0.05$, ANOVA.

Toll-like receptor 4 mediates the proinflammatory effects induced by pancreatic elastase

Toll-like receptor 4 (TLR4) has been implicated in systemic inflammation and elastase-induced systemic toxicity in experimental animals [6]. We therefore asked whether pancreatic elastase-induced NF-κB activation and proinflammatory changes might be mediated by TLR4 in cultured myeloid cells. TLR4 blocking by a specific neutralizing antibody clearly prevented NF-κB activation induced by elastase in THP-1 cells (Fig. 3A). Also, elastase-induced TNF-α secretion was mostly prevented from these cells (Fig. 3B). Finally, anti-TLR-4 antibody effectively prevented pancreatic elastase-induced increased CD11b expression in THP-1 cells (Fig. 4).

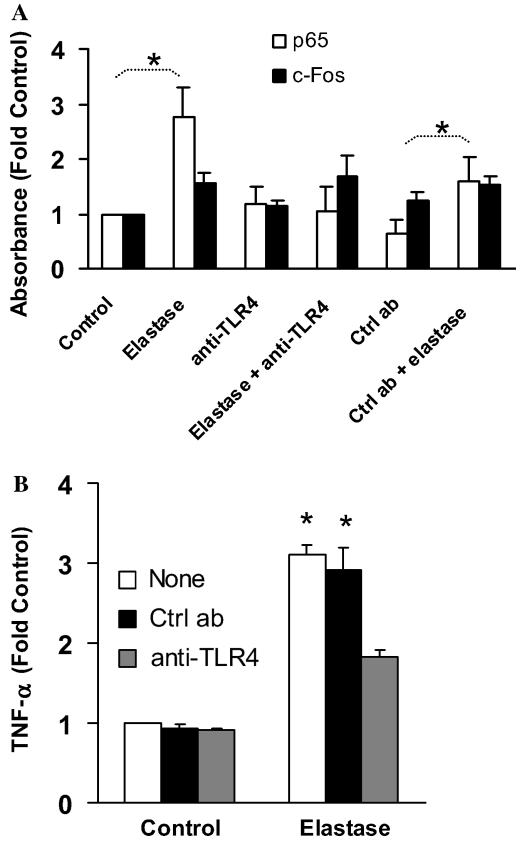

Fig. 3. The effect of TLR4 blocking on elastase-induced transcription factor activation and TNF-α secretion. (A) Transcription factor activation. THP-1 cells were preincubated (1 h) with either anti-TLR4 antibody (anti-TLR4) or control antibody (Ctrl ab) before incubation with pancreatic elastase (1 U/ml) for 24 h. Nuclear protein extracts were prepared and incubated in wells (96-well plate) coated with NF-κB or AP-1 DNA consensus sequence. Primary antibodies against transcription factor subunits (p65 for NF-κB and c-Fos for AP-1) were used and then detected by HRP-conjugated secondary antibodies and substrate. The results are expressed as fold non-treated and non-stimulated control. (B) TNF-α secretion in response to elastase stimulation. THP-1 cells were preincubated for 1 h with either anti-TLR4 antibody (anti-TLR4) or control antibody (Ctrl ab) before incubation with pancreatic elastase (1 U/ml) for 24 h. TNF-α concentration was measured from the culture media at 24 h and expressed as fold non-treated and non-stimulated control. *Denotes $p < 0.05$, ANOVA.

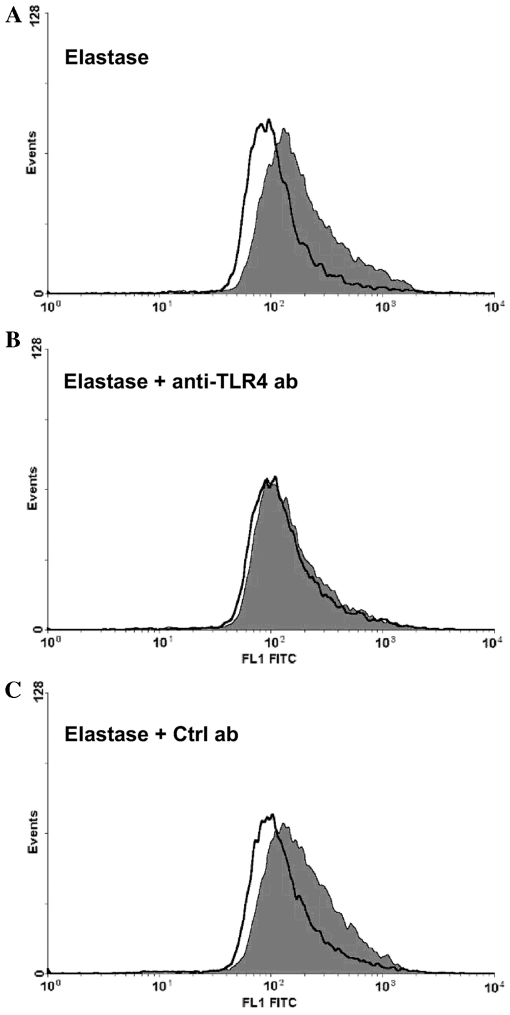

Fig. 4. The effect of TLR4 blocking on elastase-induced CD11b expression on THP-1 cells. THP-1 cells were left non-treated (A), preincubated (1 h) with either anti-TLR4 antibody (anti-TLR4) (B) or control antibody (Ctrl ab) (C) before incubation with pancreatic elastase (1 U/ml) for 72 h. Thereafter, the cells were analyzed for CD11b expression on a flow cytometer. Non-shaded area represents non-treated non-stimulated and non-treated control cells and shaded area elastase stimulated cells.

Discussion

Pancreatic elastase has been implicated in the activation of inflammatory cells during acute pancreatitis. On one hand, prematurely activated pancreatic elastase has been found in pancreatic tissue in experimental acute pancreatitis [10]. On the other hand, pancreatic elastase has been shown to activate inflammatory cells in vivo and in vitro [4,5]. Further, pancreatic elastase can be used to induce a human SIRS-like inflammatory condition with associated mortality in experimental animals

[6]. These findings suggest an important role for pancreatic elastase in the pathophysiology of severe acute pancreatitis associated systemic inflammation.

In this study, we show that pancreatic elastase acts through TLR4 and NF-κB in stimulating human inflammatory cells, therefore, sharing these signal transduction pathways with neutrophil elastase [11,12]. The activation of NF-κB in inflammatory cells by pancreatic elastase has been previously described in detail [4,5]. Here we show that pancreatic elastase activates also other transcription factors, such as AP-1 and NFAT, in addition to NF-κB. We found that MG-132 and PGA_1,which prevented NF-κB activation (p65 nuclear translocation) while inducing c-Fos nuclear translocation (AP-1 subunit), effectively prevented elastase-induced TNF-α protein secretion from THP-1 cells. These results support the central role of NF-κB in TNF-α expression. Previous studies have suggested that also p38-MAPK, not converging on NF-κB, might regulate TNF-α expression [13]. These assumptions were based on the use of SB 203580 as p38-MAPK inhibitor, which may, however, act by preventing translation of cytokine mRNA, not by preventing transcription or accumulation of mRNA [14].

Elastase induced an increased expression of CD11b in THP-1 cells in the present study. This was clearly mediated by TLR4 as it was abolished by anti-TLR4 neutralizing antibodies. However, NF-κB activity is not required for CD11b expression in THP-1 cells. We found that both MG-132 and PGA_1 alone induced an increased CD11b expression in THP-1 cells (data not shown). MG-132 and PGA_1 are known to induce transcription factors such as AP-1 [15,16].

The increased expression of leukocyte CD11b characterizes diseases with systemic inflammation and sepsis as well as severe acute pancreatitis [3]. Our findings showing that elastase induces an increased expression of CD11b in inflammatory cells further support the role of elastase in the pathophysiology of severe acute pancreatitis associated systemic inflammation.

Acknowledgments

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