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Structure of IL-17A in Complex with a Potent, Fully Human Neutralizing Antibody

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IL-17A is a pro-inflammatory cytokine produced by the newly identified Th17 subset of T-cells. We have isolated a human monoclonal antibody to IL-17A (CAT-2200) that can potently neutralize the effects of recombinant and native human IL-17A. We determined the crystal structure of IL-17A in complex with the CAT-2200 Fab at 2.6 Å resolution in order to provide a definitive characterization of the epitope and paratope regions. Approximately a third of the IL-17A dimer is disordered in this crystal structure. The disorder occurs in both independent copies of the complex in the asymmetric unit and does not appear to be influenced by crystal packing. The complex contains one IL-17A dimer sandwiched between two CAT-2200 Fab fragments. The IL-17A is a disulfide-linked homodimer that is similar in structure to IL-17F, adopting a cystine-knot fold. The structure is not inconsistent with the previous prediction of a receptor binding cavity on IL-17 family members. The epitope recognized by CAT-2200 is shown to involve 12 amino acid residues from the quaternary structure of IL-17A, with each Fab contacting both monomers in the dimer. All complementarity-determining regions (CDRs) in the Fab contribute to a total of 16 amino acid residues in the antibody paratope. *In vitro* affinity optimization was used to generate CAT-2200 from a parental lead antibody using random mutagenesis of CDR3 loops. This resulted in seven amino acid changes (three in VL-CDR3 and four in VH-CDR3) and gave an approximate 30-fold increase in potency in a cell-based neutralization assay. Two of the seven amino acids form part of the CAT-2200 paratope. The observed interaction site between CAT-2200 and IL-17A is consistent with data from hydrogen/deuterium exchange mass spectrometry and mutagenesis approaches.

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Abbreviations used: CDR, complementarity-determining region; scFv, single-chain variable fragment; V_H, variable heavy; V_L, variable light; SPR, surface plasmon resonance; HTRF, homogeneous time-resolved fluorescence; PDB, Protein Data Bank; H/D, hydrogen/deuterium; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; NEAA, nonessential amino acids; MR, molecular replacement.

Introduction

IL-17A is one of six known members of the IL-17 cytokine family (IL-17A-F).[1] It is a secreted homodimeric glycoprotein with a molecular mass of ~35 kDa.[2] IL-17 family members play important and distinct roles in adaptive immune responses. They mediate their effects through the IL-17 receptor family, of which there are five related members (IL-17RA–IL-17RE; reviewed by Shen and Gaffen[3] and Gaffen[4]). Both IL-17A and IL-17F can bind to either IL-17RA or IL-17RC, and it has been proposed that these colocalize at the cell surface and function as heterodimeric receptors.[5] It has also been shown that IL-17A and IL-17-F can form functional heterodimers in human T-cells and can induce neutrophilia in a murine adoptive transfer model.[6,7]

Recent studies have identified Th17 cells as a unique and distinct $CD4^+$ T-cell lineage that is defined by the production of IL-17A, IL-17F, IL-6, tumor necrosis factor, granulocyte–macrophage colony-stimulating factor, IL-21, IL-22, and IL-26 (reviewed by Shen and Gaffen[3] and Bettelli *et al.*[8]). Th17 cells are believed to have evolved as an arm of the adaptive immune system and have a critical role in maintaining inflammatory responses, a role previously ascribed to Th1 cells. Th17 cells are therefore emerging as strong candidates for drivers of autoimmune disease.[9]

IL-17A is not widely expressed in humans and is only found at very low concentrations, specifically in areas populated by Th17 cells. Interestingly, IL-17A is expressed in disease compartments in a range of autoimmune diseases (reviewed by Witowski *et al.*[10]) such as rheumatoid arthritis,[11–13] multiple sclerosis,[14,15] psoriasis,[16] and inflammatory bowel disease.[17] *In vivo* studies have shown that IL-17A has a distinct and critical role in driving both the early initiation phase and the late progression phase of disease in a number of preclinical models of rheumatoid arthritis.[18]

Given these recent findings, it is not surprising that Th17 cells and members of the IL-17/IL-17 receptor family have become the focus of intense investigation and have been viewed as potential targets for therapeutic intervention. One group has recently developed an anti-IL-17 antibody that is currently in early clinical studies.[19]

The reported crystal structure of IL-17F (which has a 50% sequence identity to IL-17A) presents a disulfide-linked homodimeric glycoprotein that adopts a classical cystine-knot fold found in the transforming growth factor β, bone morphogenetic protein, and nerve growth factor superfamilies; however, it lacks the classical disulfide bond responsible for the canonical knot[20] and instead has two serines replacing the cysteine residues. All members of the IL-17 family lack the cysteine residues required to form the knot, but instead have conserved serines.

IL-17A is the most intensively studied member of the IL-17 cytokine family, yet no experimentally determined structure has been published to date. Here, we describe the generation of CAT-2200, a potent, fully human neutralizing monoclonal antibody to IL-17A, and reveal the crystal structure of IL-17A in complex with a Fab fragment of this antibody. This reveals the definitive epitope and paratope of the antibody–antigen complex, fully satisfying the experimental intention. It is interesting to examine the structural context of the mutations that result in the improved potency of the CAT-2200 antibody in relation to the parental clone and to speculate which parts of the IL-17A structure might be involved in receptor binding.

Results

Isolation of the anti-IL-17A antibody CAT-2200

IL-17A binding antibodies were isolated from a large phage library displaying human single-chain variable fragments (scFv)[21] by panning selections on recombinant human IL-17A. A panel of scFv isolated from these selections was identified by their ability to neutralize the binding of recombinant IL-17A to purified IL-17RA·Fc fusion (receptor–ligand binding assay), with IC_{50} values ranging from 4 nM to >1000 nM (data not shown). These scFv were reformatted as full-length IgG1 molecules and tested for neutralization of human IL-17A in a functional cell assay measuring the release of IL-6 from HT1080 cells in response to IL-17A. The most potent lead antibody identified from the cytokine release assay, TINA12, neutralized the activity of IL-17A with an IC_{50} of 23 nM.

TINA12 was optimized for affinity by a randomized mutagenesis of the variable heavy (V_H) and the variable light (V_L) complementarity-determining region (CDR) 3. V_H CDR3 and V_L CDR3 were mutated separately to generate a number of libraries. scFv phage libraries containing CDR3 variants of the lead antibody were subjected to multiple rounds of affinity-based solution-phase phage display selections. A panel of optimized scFv was isolated from these selections through their improved ability to neutralize the binding of IL-17A to IL-17RA relative to the parental TINA12 antibody. The optimization process identified scFv antibodies with IC_{50} values of 0.6–12 nM in the receptor–ligand binding assay (data not shown). These optimized scFv were reformatted as IgG1 (λ light chain) and tested for neutralization of human IL-17A on HT1080/IL-6 release assay. The V_H and V_L chains from several of the most potent antibodies were recombined, and the most potent recombined antibody was then reverted by mutagenesis to the closest human germline sequence (genes VH3-23 and VL6-6a) in the VBASE database,[22] generating the anti-IL-17A antibody CAT-2200. Any framework residue that was reverted back to germline was assayed to ensure that it did not affect antibody affinity. CAT-2200 neutralized IL-17A with an IC_{50} of 0.8 nM in the HT1080/IL-6 assay, representing a

Table 1. A comparison of lead antibody (TINA12) to optimized antibody (CAT-2200)

	Light chain CDR3									Heavy chain CDR3									Affinity K_d (nM)	Potency IC_{50} (nM)	
	89	90	91	92	93	94	95	96	97	95	96	97	98	99	100	100a	101	102	IgG	IL-17A (*E. coli*)	IL-17A (mammalian)
TINA12	Q	S	Y	D	D	S	S	V	V	D	L	I	W	G	V	A	G	S	13.5	23	ND
CAT-2200	Q	**T**	Y	D	**P**	**Y**	S	V	V	D	L	I	**H**	G	V	**T**	**R**	**N**	2.1	0.8	0.13

The numbering of amino acids is performed in accordance with Kabat *et al.*[23] Affinities were measured with BIAcore by immobilizing the antibody and using standard procedures in accordance with the manufacturer's instruction. Potency was determined by the ability to inhibit the production of IL-6 from HT1080 cells stimulated with recombinant IL-17A.

~30-fold improvement over TINA12. The seven CDR3 amino acid changes (three in V_L and four in V_H) that result in the increased potency of CAT-2200 compared that of the TINA12 parental clone are shown in Table 1.

The binding affinity of both TINA12 and CAT-2200 for IL-17A was measured using surface plasmon resonance (SPR). The increase in apparent affinity upon optimization of TINA12 to CAT-2200 was approximately 6-fold for the IgG1 molecule, with almost all of the improvements caused by a decrease in off-rate.

Functional characterization of CAT-2200

The functional activity of CAT-2200 was assessed against a variety of sources of IL-17A on a biochemical assay and acting on different cell types.

The antibody potently inhibited the binding of IL-17A to IL-17RA-Fc in homogeneous time-resolved fluorescence (HTRF) format (Fig. 1a). To assess antibody functional activity in a disease-relevant cell system, we investigated the effects of CAT-2200 on recombinant IL-17A-induced IL-8 responses in human primary chondrocytes. *Escherichia-coli-*

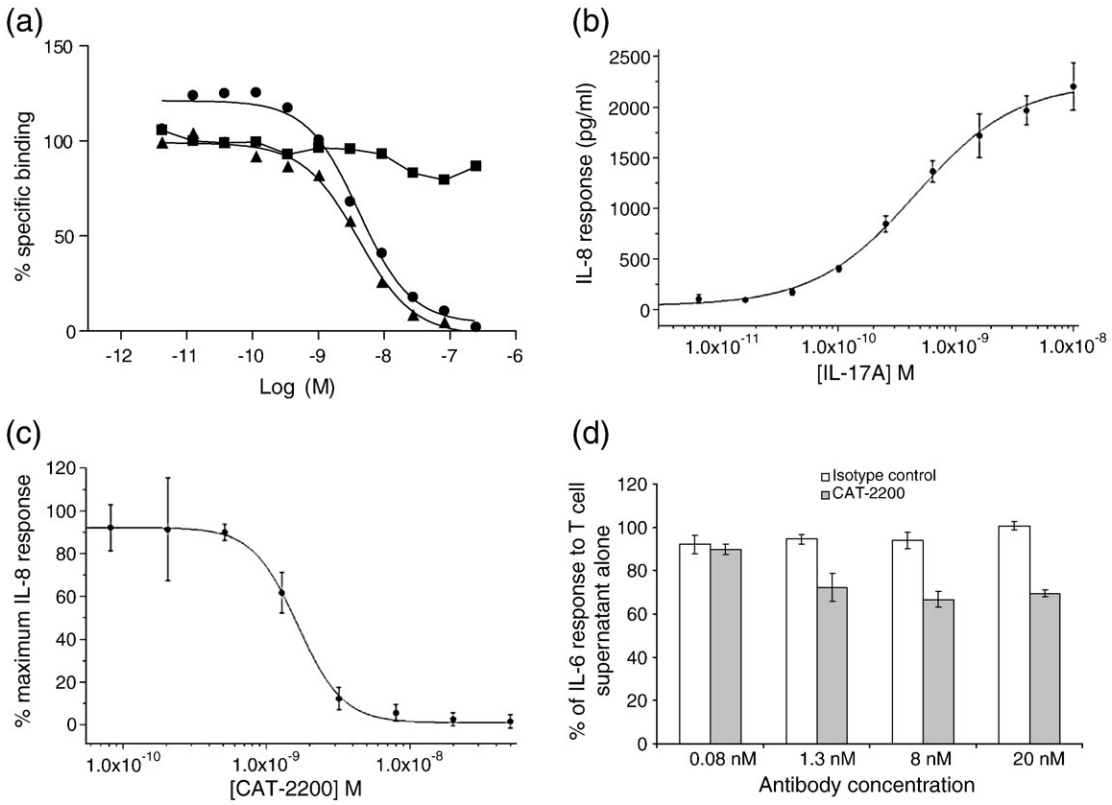

Fig. 1. IL-17A-induced responses and their inhibition by CAT-2200. (a) Inhibition of FLAG-tagged IL-17A binding to IL-17RA-Fc using HTRF. Triangles, untagged *E.-coli*-derived IL-17A; circles, CAT-2200; squares, isotype control antibody. (b) Recombinant *E.-coli*-derived IL-17A induced IL-8 release from primary human chondrocytes. Mean±SD for one donor ($n=3$). $EC_{50}=0.46$ nM. (c) CAT-2200-mediated inhibition of *E. coli* IL-17A (2 nM) induced IL-8 release from primary human chondrocytes. Mean±SEM for three donors. Mean $IC_{50}=1.56$ nM. (d) CAT-2200-mediated inhibition of T-cell-derived IL-17A induced IL-6 release from HT1080 cells compared to isotype control. Mean±SEM for experiments using different T-cell supernatants.

derived IL-17A generated a dose-dependent increase in IL-8 production from primary human chondrocytes with an EC_{50} of 0.46 nM. CAT-2200 inhibited this response with an IC_{50} of 1.56 nM (Fig. 1b and c).

In a second system, recombinant IL-17A derived from a mammalian cell line was shown to induce the production of IL-6 from HT1080 cells with an EC_{50} of approximately 0.3 nM. This effect could be inhibited by CAT-2200 with an IC_{50} of 8 nM when using 1 nM IL-17A.

The neutralizing activity of CAT-2200 against native IL-17A derived from human T-cells was also analyzed (Fig. 1d). T-cells were cultured under conditions enhancing IL-17A production.[24] Supernatants contained IL-17A plus other mediators, including IL-6 and tumor necrosis factor α, which may synergize with IL-17A. T-cell supernatants from three donors induced IL-6 release from HT1080 cells, and the effect of CAT-2200 on IL-6 levels was assessed. The maximum inhibition with CAT-2200 was 30%, which was maintained at concentrations of 8 nM and above. Isotype control IgG showed no effect on IL-6 levels at this concentration. Partial inhibition almost certainly reflects the presence of IL-6 in the T-cell-conditioned medium, as well as other cytokines that would have been produced under the conditioned medium of stimulated T-cells. Thus, partial inhibition is likely to represent that portion that is a result of the IL-17A component. This suggestion is also supported by the observation that the potency of CAT-2200 in this assay is very similar to that in Fig. 1c when recombinant IL-17A is used. Thus, CAT-2200 is able to neutralize the activity of a native T-cell-derived source of IL-17A.

The cross-reactivity of CAT-2200 to different IL-17 family and species variants was assessed by the ability of these proteins to inhibit the binding of antibody to recombinant human IL-17A derived from the mammalian HEK293/EBNA cell line. The rank order of the binding of CAT-2200 to different species variants was human>cynomolgus>canine, with no observed binding to murine IL-17A. In addition, CAT-2200 showed no binding to human IL-17 family members B–E. Some weak binding (20% inhibition at 1 μM) to IL-17F was observed at the highest concentration of IL-17F tested.

In summary, we have isolated an antibody that can potently neutralize the effects of recombinant and native human IL-17A on a number of cell systems. Furthermore, the antibody does not cross-react with the other IL-17 family members B–E; however, it does recognize IL-17F, albeit with a low potency.

Crystal structure of the IL-17A/CAT-2200 complex

Overall complex structure

The structure of IL-17A/CAT-2200 was refined to 2.6 Å resolution with R/R_{free} of 21.2%/26.4%. The asymmetric unit in the crystal contains two complex molecules, each with two Fab fragments bound to the IL-17A dimer. Hence, the final model of six molecules present in the asymmetric unit comprises 2043 amino acid residues. Of these, 1720 residues are located within four molecules of the antibody Fab fragment (heavy chains H, I, J, and K, and light chains L, M, N, and O). The remaining 323 residues are found in the two IL-17A homodimers (chains A/B and C/D). More than 99% of all residues of the complex structure were found in the most favored and additionally allowed regions of the Ramachandran plot.[25] Of the remaining residues, 0.2% fall into the generously allowed regions and 0.5% fall into the disallowed regions.

The crystal structure shows that, in the antibody complex, each IL-17A dimer is sandwiched between two Fab fragments (Fig. 2), generating two equivalent IL-17A/Fab interaction sites related by the IL-17A dimer symmetry. The buried surface area per interface is around 760 $Å^2$. To our surprise, in the complex structure, as illustrated in Fig. 2, the lower portion of the IL-17A dimer is disordered, indicating that it is flexible and adopts different orientations throughout the crystal such that electron density is averaged out and is not visible. Thus, it is not possible to build a model for this part of the IL-17A dimer with the current data. Two polypeptide segments are affected: 34 or 35 amino acid residues at the N-termini and 9 or 11 amino acids starting at residue 100 or 101. The two independent copies reveal the same disorder, differing in extent by just a single residue. In the crystal, lattice interactions are mediated through the Fab molecules only. There is ample room in the lattice for the entire IL-17A molecule to be present in a conformation equivalent to that seen in IL-17F. Hymowitz *et al.* described IL-17F as a 'garment' with a 'collar,' 'sleeves,' a 'body,' and a 'skirt'.[20] In the IL-17A structure presented here, it is the skirt that is disordered. The epitope interaction sites are at the collar and sleeves of the structure.

An overlay of CAT-2200 Fab with Protein Data Bank (PDB) entry 1AQK, demonstrating that the CDRs are in canonical conformation (with the exception of VH-CDR3), is shown in Fig. 3.

Structure of IL-17A and comparison to IL-17F

The structure of IL-17A and structural alignment with IL-17F are shown in Fig. 4a and b. Within the IL-17 cytokine family, IL-17A is the closest homologue to IL-17F, with a 50% sequence identity. The structure of IL-17F was solved by Hymowitz *et al.*[20] (PDB entry 1JPY), unexpectedly revealing a cystine-knot fold.[26] The IL-17A dimer can be superposed onto the IL-17F dimer with a root-mean-square deviation (r.m.s.d.) of 1.1 Å for 132 C^{α} positions. The sequence identity for the overlaid portion of the polypeptides is 64%, higher than the overall sequence identity between the two molecules. This is not surprising; apparently, the ordered part of the molecule is the more conserved part. Each of the protomers of IL-17A present in the asymmetric unit of the crystal lattice can be superimposed onto each

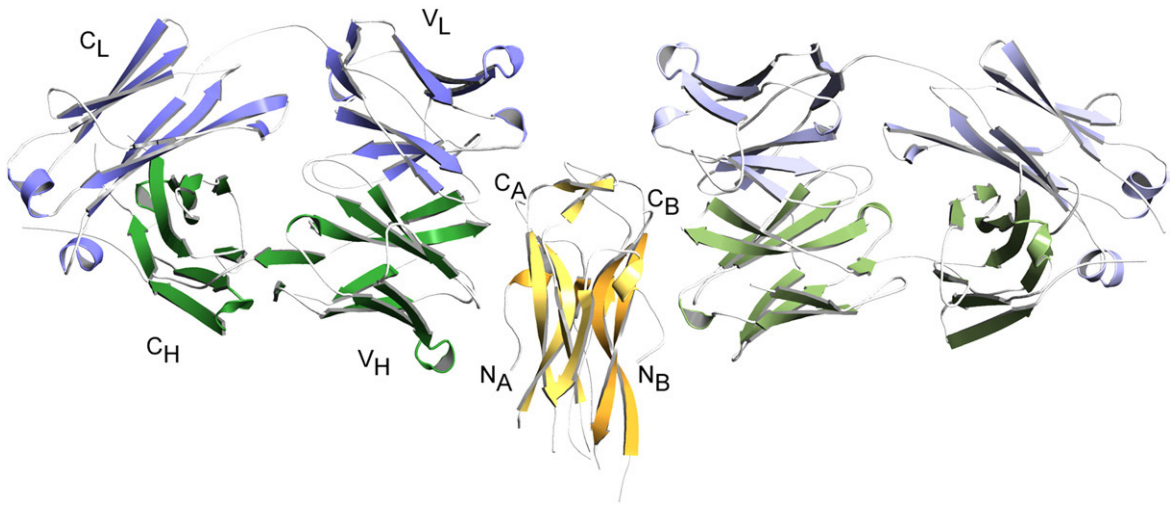

Fig. 2. Overall structure of the IL-17A/Fab complex. This and all other molecular illustrations in this work were prepared using PyMOL (http://www.pymol.org). The IL-17A homodimer is shown with the two molecules of the dimer in pale and dark yellow. The Fab fragments are shown with the light chain in blue and with the heavy chain in green. The constant and V_H and V_L domains are labeled. The two interaction sites are equivalent, related by IL-17A dimer symmetry. The lower portion of the IL-17A dimer is not visible on the electron density map and does not form part of this model. The N-termini and C-termini of monomers A and B are indicated.

other using 74–79 α-carbon atoms, giving an r.m.s.d. of between 0.32 Å and 0.48 Å.

IL-17A has a homodimeric assembly. Each subunit is formed by a set of two pairs of anti-parallel β-strands (β1/β2 and β3/β4). A short helix from Asp42 to Arg46 is the only helical feature. The IL-17A monomer has the same cystine-knot architecture identified in the crystal structure of IL-17F. The classical cystine knot is formed by the unique arrangement of six cysteine residues. In the structure of IL-17A, Cys71 and Cys121, as well as Cys76 and Cys123, connect β-strands 2 and 4 to form one part of the knot. A true cystine knot requires an additional disulfide to penetrate the ring formed, but cysteine-to-serine replacements at positions 49 and 89 of the amino acid sequence of IL-17A

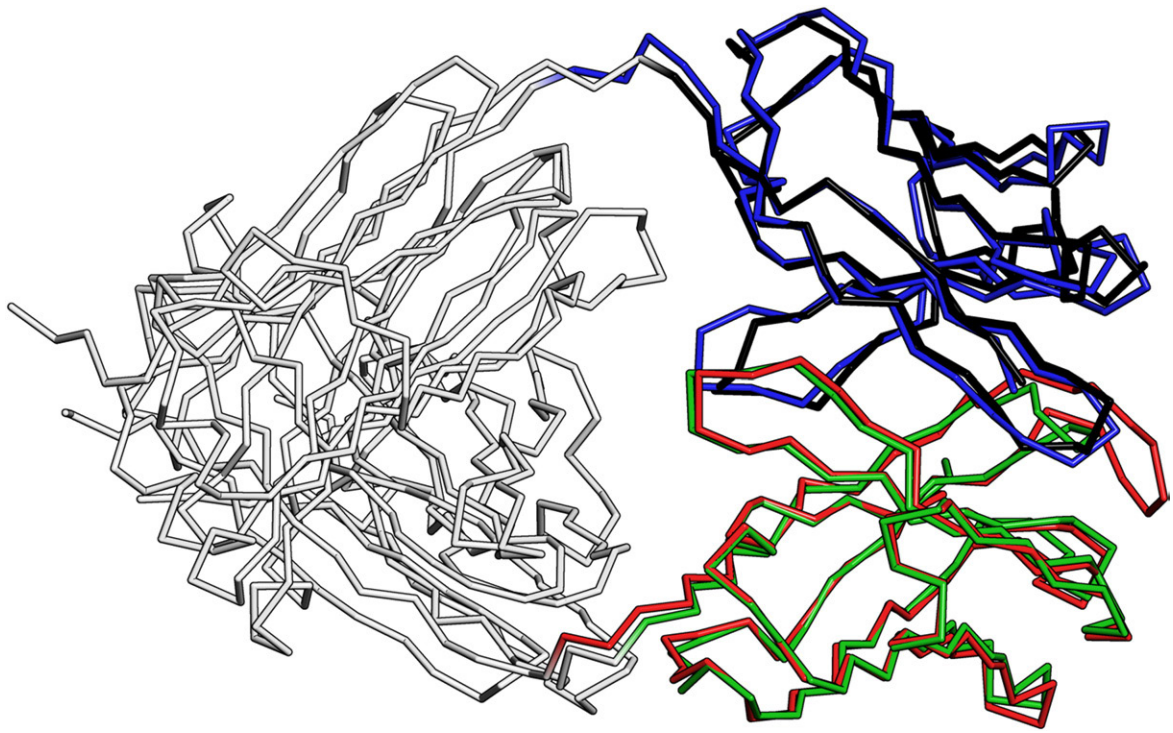

Fig. 3. Overlay of CAT-2200 Fab with PDB entry 1AQK. The constant domains are shown in white. PDB entry 1AQK is shown with the light chain in black and with the heavy chain in red. CAT-2200 is shown with the light chain in blue and with the heavy chain in green.

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