results of Liu *et al.*[4,5]. However, Flag–TALL-1 elutes as a trimer of 62K (M_r of the Flag–TALL-1 trimer is 58K) that is stable for several months. Liu *et al.*[4] used a western blot of fractions from size-exclusion chromatography to resolve both trimeric and oligomeric forms of Flag–TALL-1. However, the latter species eluted in the void volume of a Superdex-200 gel-filtration column, which is consistent with either the 60-mer or non-specific aggregates.

We also tested whether the Flag tag prevents the formation of 60-meric TALL-1 and whether the His tag causes trimeric TALL-1 to form 60-mers. We removed the TALL-1 tags using factor Xa and analysed the size of the digested products. We found that removing the Flag tag did not alter TALL-1's oligomerization state. Removing the His tag caused TALL-1 to convert from a 60-mer to a trimer (Fig. 1a). This suggests that the His tag promotes formation of non-native oligomers.

The pH dependence of the trimer-to-60-mer transition[4] alters with the ionization of the imidazole ring of histidine (p*Ka* 6.0–7.0). Whereas a neutral amino-terminal tag (that is, when the histidine ring is not ionized because the buffer pH is above its p*Ka*) promotes 60-mer formation, a positively charged His tag (buffer pH below the histidine p*Ka*) or a negatively charged Flag tag does not induce TALL-1 oligomerization. Consistent with this, untagged TALL-1 is trimeric (Fig. 1a).

We tested viability (Fig. 1b) and B-cell proliferation (data not shown) and confirmed that trimeric TALL-1 is biologically active. It is not surprising that trimers and 60-mers are both active because oligomerization does not always affect biological activity[8]; alternatively, interaction with membrane-bound TALL-1 receptors may force dissociation of 60-mers, such that both species give a similar biological response.

Our results indicate that native TALL-1, in common with all members of the TNF-ligand superfamily, is trimeric and biologically active. We have shown that introduction of an amino-terminal histidine tag affects the oligomerization state of TALL-1, and we warn generally against the use of fusion tags that might perturb the physical chemical properties of the host protein.

Eugene A. Zhukovsky*, Jie-Oh Lee†, Michael Villegas*, Cheryl Chan*, Seung Chu*, Cameron Mroske*

**Xencor, Monrovia, California 91016, USA*
e-mail: ezhukovsky@xencor.com
†Department of Chemistry and School of Molecular Science, KAIST, Daejeon 305–701, Korea

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Liu et al. reply — Our conclusion that a 60-meric soluble-TALL-1 cluster is the functional unit of this ligand[1,2] is based on several lines of evidence.

We have shown that soluble TALL-1 (sTALL-1; residues 134–285) with a Flag tag, secreted from 293 cell lines, spontaneously forms a virus-like cluster in the culture medium under physiological conditions[1]. Also, Flag-tagged proteins dissociate into trimers and monomers at low pH, ruling out the possibility of random aggregation that is suggested by Zhukovsky and colleagues.

The pH dependence of the sTALL-1 structure is consistent with the presence of a histidine residue in the flap region[1], the removal of which prevents the oligomerization of sTALL-1 and abolishes the activity of the ligand in transfection and in B-cell-stimulation assays[1]. However, this truncated version of the molecule still binds to its receptor[1], indicating that the missing flap region is not involved in receptor binding[2]. The structure of the truncated molecule is the same as that of native sTALL-1, apart from the missing flap region[2].

It is true that some His6-tagged proteins are susceptible to aggregation caused by divalent metals. We tested whether this could be the case for our His6-tagged sTALL-1 protein by adding 100 mM EDTA, pH 7.5, at the different stages of purification. We found that it did not affect oligomerization. Note also that the His6 tag is far away from the trimer–trimer association region in our structure[1].

To prevent any perturbation of the oligomerization properties of sTALL-1 by the His6 or Flag tags, we overexpressed an untagged version of sTALL-1 (residues 134–285) in *Escherichia coli*. This sTALL-1 (prepared and characterized with the help of Zhongzhou Chen) was purified on a Q-Sepharose column in buffer (20 mM Tris-HCl) at pH 7.2, concentrated in an Amicon Ultra unit (Millipore) and applied to Superdex-200 (150 mM NaCl, 20 mM Tris-HCl) at pH 8.0. sTALL-1 emerged as a multimer in the void volume (data not shown). The buffer containing this sample was then adjusted to pH 6.0, equilibrated for 2 hours and reapplied to the Superdex-200 column in the same buffer at pH 6.0.

The elution profile consisted of a single sharp peak: the molecular size of the material in this fraction was estimated from the elution positions of three M_r standards (blue dextran, 2,000,000 (2,000K); ferritin, 440K; and ovalbumin, 43K) under the same conditions. The pure sTALL-1 protein eluted at a position corresponding to a trimer. Amino-terminal sequencing analysis confirmed that this was the correct protein (data not shown).

Other crystal structures[3,4] reported for trimeric sTALL-1 were obtained with proteins that had been crystallized at low pH (pH 4.5 and 6.0, respectively), conditions under which the trimer should be the only species present[1]. Zhukovsky *et al.* used native sTALL-1, with and without tags, in their assays. As these should both be transformed to 60-mers under physiological conditions, it is not surprising that these forms are active. If, by contrast, they had used a flapless version of the protein, which does not oligomerize, we predict that it would have been inactive. We contest that neither the His6 tag nor the Flag tag promotes or blocks the formation of 60-mers by sTALL-1 and that it is the 60-mer, rather than a trimer or some other oligomer, that is the functional unit.

Other data indicate that further oligomerization of ligand trimers (multiple valences) or mulitple trimers are required to activate the signal-transduction pathway of the TNF superfamily. For example, TNF receptors can oligomerize on the cell surface without bound ligand[5]. TNF-receptor-associated factors (TRAF), which mediate downstream signal transduction by the TNF family, exist as trimers before TNF ligands are recruited to its receptors[6]. Ectodysplasin ligand A1 (EDA-A1) and A2 (EDA-A2) require a collagen-like region for their proper function, which suggests that two dimers or higher-order clusters are needed for the activation of these ligands[7]. Combining with our results with sTALL-1, it is reasonable to suggest that one trimer of the ligand is not enough to trigger signal transduction for TNF family members. Multiple trimers (including trimers in different oligomerization states of increased concentration and valence and hence increased avidity) or a local accumulation of trimers (increasing in concentration alone) on the cell surface may be required for the optimal recruitment of a cluster of receptors to activate signal transduction. This is similar to the supermolecular activation cluster phenomenon of T-cell activation that occurs after binding to antigen-presenting cells[8].

Xia Hong*, John Kappler*†, Yingfang Liu*, Liangguo Xu*, Hong-Bing Shu*, Gongyi Zhang*

**Integrated Department of Immunology, National Jewish Medical and Research Center, †Howard Hughes Medical Institute, and Department of Pharmacology, Biomolecular Structure Program, School of Medicine, University of Colorado Health Science Center, Denver, Colorado 80206, USA*
e-mail: zhangg@njc.org

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