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Short communication

Tandem immunoprecipitation approach to identify HIV-1 Gag associated host factors

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HIV-1 Gag by itself is able to assemble and release from host cells and thus serves as a simplified model to identify host factors involved in this stage of the HIV-1 life cycle. In this study, a tandem immunoprecipitation approach is taken to immunoprecipitate Gag-interacting host proteins from transfected 293T cells. It is demonstrated that with the tandem immunoprecipitation method Gag-interacting host factors can be precipitated more efficiently than by single-step immunoprecipitation. Gag proteins are found to interact with multiple RNA-binding proteins such as hnRNPs, nucleolin, EF1a and ribosomal proteins. Such interactions are mediated by cellular RNAs and the Gag Nuclear Capsid (NC) domain. Deletion of the NC domain results in removal of most of the RNA-binding proteins, as well as a reduction of the Gag releasing capability, which can be restored by replacing the deleted NC domain with another multimerization motif. Importantly, interactions between Gag and host factors are relevant functionally, as evidenced by significantly increased nucleolin protein in the cytoplasm where it is recruited into the Gag complex, and enhanced Gag release when nucleolin is over-expressed.

Host factors that are involved in HIV assembly and release could be important targets for novel HIV therapeutics. Pioneering work has established the Endosomal-Sorting Complexes Required for Transport (ESCRT) system as part of the cellular machinery that is hijacked by HIV Gag proteins for their budding out of the host cells (Bell and Lever, 2013). Recently, it was demonstrated that tetherin, an interferon-induced host membrane protein, inhibits viral release by targeting the host cell-derived lipid bilayer (Perez-Caballero et al., 2009). Over the past decade there have been considerable advances in the field of HIV-1 assembly and release; however, it is believed that more host factors remain to be discovered for a better understanding of this process.

Various techniques have been employed to identify such host factors, including the yeast two-hybrid system (VerPlank et al., 2001) and functional genetic screening (Brass et al., 2008). In a recent proteomic study, hundreds of host proteins were identified residing inside of HIV-1 virions (Linde et al., 2013). Functionally, these cellular proteins may facilitate viral assembly and release, or they may be incorporated by the virus to prepare for the next cycle of infection. In addition, the presence of many other HIV proteins complicates the interpretation of the mechanism by which cellular proteins are recruited into HIV virions. To avoid these complexities, in this study HIV Gag is explored as a simplified model to identify host factors involved in HIV assembly and release, based on the fact that HIV Gag by itself is able to assemble and release from host cells.

A tandem immunoprecipitation approach was taken to identify Gag-associated host proteins (Fig. 1a). Two tags, HA (YPYDVPDYA) and Flag (DYKDDDDK), were attached sequentially to the C-terminus of Gag to generate a Gag-HA-Flag construct. 293T cells were transfected with Gag-HA-Flag, and a stable cell line with modest expression of Gag proteins was established. Cells were lysed with Tris buffer containing 1% NP40 and 150 mM NaCl. Cell lysates pre-cleared with protein A were first immunoprecipitated with beads loaded with an anti-Flag M2 antibody. For single precipitation, the beads were washed and denatured with sample buffer directly. For tandem precipitation, the beads were subsequently eluted with 3x Flag peptide dissolved in Tris buffer. The eluent was subjected to a second round of immunoprecipitation with beads loaded with an anti-HA antibody. The beads were washed, denatured with sample buffer, and analyzed on a 4–20% gradient gel. Individual bands visualized by silver staining were subjected to proteomic analysis by mass spectrometry (Fig. 1a). As shown in Fig. 1b, two rounds of immunoprecipitation were more efficient in precipitating Gag-associated proteins than a single immunoprecipitation step. Most of the Gag-associated proteins identified

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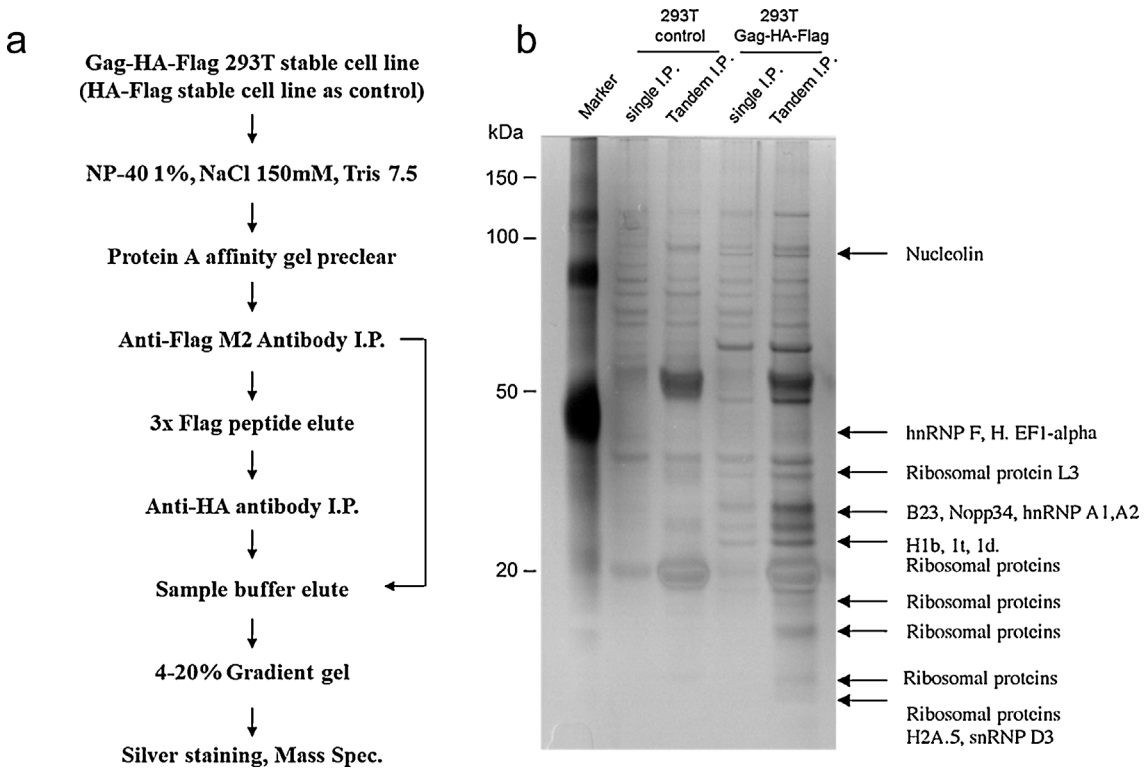

Fig. 1. Method and results of HA-Flag tandem immunoprecipitation in identification of intracellular proteins associated with HIV-1 Gag. (a) Schematic representation of tandem immunoprecipitation for proteomic analysis by mass spectrometry. 293T cells stably expressing Gag-HA-Flag protein were lysed with NP-40-containing buffer. Cell lysates were first cleared with protein A gel, and then subjected to immunoprecipitation with beads loaded with anti-Flag M2 antibody. For single immunoprecipitation, the beads were washed and denatured directly with sample buffer. For tandem immunoprecipitation, the beads were subsequently eluted with 3x Flag peptide dissolved in Tris buffer. Eluent was subjected to a second round of immunoprecipitation with beads loaded with anti-HA antibody. The beads were washed, denatured with sample buffer, and analyzed on a 4–20% gradient gel. Individual bands visualized by silver staining were subjected to proteomic analysis by mass spectrometry. (b) Immunoprecipitated proteins identified by mass spectrometry are marked to the right of the gel. Four samples are shown: 293T cells expressing control HA-Flag or Gag-HA-Flag proteins subjected to either single or tandem immunoprecipitation. *Abbreviation*: I.P. = immunoprecipitation.

are involved in RNA binding. These include nucleolin, hnRNPA1, hnRNPA2, hnRNPF, hnRNPH, EIF1a, and multiple ribosomal proteins (Fig. 1b).

Because of the RNA-binding capabilities of both Gag and many Gag-associated proteins, it was necessary to determine whether the interactions were indeed mediated by RNAs (Waheed and Freed, 2012). As shown in Fig. 2a, samples from tandem immunoprecipitation of cells expressing Gag-HA-Flag or a control were analyzed on an RNA gel, which revealed the presence of abundant cellular RNAs, including ribosomal RNAs (100–200 nt) and others with high molecular weights, in the Gag-associated complex. The RNAs involved did not seem to be random; for example, tRNAs, which are abundant in the cytoplasm, are absent in the Gag complex (Fig. 2a).

To investigate if the RNA-binding NC domain of the gag protein may be involved in the recruitment of Gag-interactors, constructs with modifications of the Gag NC domain were generated. In one construct, Gag-NC-del-HA-Flag, the NC domain was removed; in another construct, Gag-GCN-HA-Flag, the NC domain was replaced with the GCN motif, a leucine zipper binding domain that has been shown to mulitimerize proteins (O'Shea et al., 1991) (Fig. 2b). Sequential immunoprecipitation with either mutated construct revealed that most of the Gag-associated proteins could not be immunoprecipitated as they were with the complete Gag-HA-Flag construct (Figs. 2c and 1b). This suggests that most of these RNA-binding proteins interact with the NC domain of Gag, and that this interaction is mediated by RNA. Gag release efficiencies were compared among cells expressing wild-type Gag, Gag with NC deletion, and Gag with GCN. As shown in Fig. 2d, deletion of the NC domain made Gag release very inefficient, and this could be rescued by GCN replacement. This result suggested that the interaction between Gag (NC domain) and host factors (RNA and RNA-binding proteins) plays a role similar to that of GCN, which is to facilitate the multimerization of Gag proteins.

To understand better such interactions, their effects on both Gag and host factors were investigated. Nucleolin is one of the proteins identified in this study. It was thought to be a protein localized normally in the nucleoli, where it plays a role in the maturation of ribosomes (Abdelmohsen and Gorospe, 2012). Gag, however, is known to be assembled in the cytoplasm. This raised the possibility that nucleolin can be localized in the cytoplasm when Gag is present. Immunofluorescence studies did not yield images with sufficient resolution to make a definitive conclusion, but did suggest that in the presence of Gag, nucleolin signal in the cytoplasm was increased (data not shown). A biochemical approach was thus taken in an effort to confirm this hypothesis. Cytoplasmic and nuclear fractions were prepared carefully from cells expressing control or Gag-HA-Flag and analyzed by Western blot. The purities of the cytoplasmic and nuclear extracts were demonstrated in Fig. 3a. PKCa was used as a marker indicating the presence or contamination of cytoplasmic proteins, and LaminB1 as a marker for nuclear proteins. Nuclear fractions became cleaner after multiple washes, as shown by the presence of LaminB1 but not PKCa protein in the Nuc-2 fraction. In control cells, most of the nucleolin protein was localized in the nucleus, with only a small fraction in the cytoplasm. When Gag protein was expressed, there was a significant increase of nucleolin in the cytoplasm (Fig. 3a). This demonstrated that presence of Gag protein increases the level of cytoplasmic nucleolin.