Oligomerization of the influenza virus polymerase complex in vivo

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The influenza virus polymerase is a heterotrimer formed by the PB1, PB2 and PA subunits and is responsible for virus transcription and replication. We have expressed the virus polymerase complex by co-transfection of the subunit cDNAs, one of which was tandem affinity purification (TAP)-tagged, into human cells. The intracellular polymerase complexes were purified by the TAP approach, involving two affinity chromatography steps, IgG–Sepharose and calmodulin–agarose. Gel-filtration analysis indicated that, although most of the purified polymerase behaved as a heterotrimer, a significant proportion of the purified material migrated as polymerase dimers, trimers and higher oligomers. Co-purification of polymerase complexes alternatively tagged in the same subunit confirmed that the polymerase complex might form oligomers intracellularly. The implications of this observation for virus infection are discussed.

The influenza A viruses belong to the family Orthomyxoviridae and contain a segmented genome formed by eight single-stranded RNA molecules of negative polarity (reviewed by Elton et al., 2005; Neumann et al., 2004; Palese & Shaw, 2006). The molecular machineries responsible for transcription and replication of the virus genome are the ribonucleoprotein (RNP) complexes, each one containing one RNA segment associated to nucleoprotein (NP) monomers and the polymerase complex (Klumpp et al., 1997; Martín-Benito et al., 2001; Ortega et al., 2000). The enzyme responsible for virus RNA synthesis is the RNA polymerase, a heterotrimer composed of the PB1, PB2 and PA subunits. PB1 is the structural core of the complex (Díged et al., 1989) and contains the polymerase and endonuclease activities. PB2 is responsible for cap recognition during transcription initiation and PA is a phosphoprotein with protease activity involved in RNA replication (reviewed by Elton et al., 2005; Palese & Shaw, 2006). The virus polymerase complex is a very compact structure (Area et al., 2004; Torreira et al., 2007) and mutations in the various subunits may alter either transcription or replication (Fodor et al., 2002; Gastaminza et al., 2003; Hará et al., 2006).

The first step in virus gene expression is the primary transcription of virion RNPs. The RNP-associated viral polymerase recognizes cellular pre-mRNAs and cleaves them at 10–15 nt downstream of the cap, thereby generating cap-containing primers for virus mRNA synthesis (Bouloy et al., 1978; Krug et al., 1979). Virus transcription is terminated by reiterate copy of an oligo-U signal located close to the 5′ terminus of the template, which results in the synthesis of a 3′-terminal poly(A) tail (Poon et al., 1999; Robertson et al., 1981). On the contrary, replication of viral RNPs does not require capped primers and occurs by the synthesis of a complementary RNA (cRNA) intermediate, which is a complete copy of the virion RNA (vRNA) (Hay et al., 1982), and serves as template for the generation of progeny vRNAs. Both vRNAs and cRNAs are encapsidated into RNP structures during replication (reviewed by Elton et al., 2005).

In addition to the RNP-associated polymerase, soluble polymerase complexes have been detected in infected cells (Detjen et al., 1987) and the interaction of cellular proteins with the polymerase complex has been described (Deng et al., 2006; Mayer et al., 2007; Momose et al., 2002; Naide et al., 2007) (N. Jorba and others, unpublished data). To characterize these soluble intracellular polymerase complexes we have addressed their expression and purification from human cells. Here we show that dimers, trimers and higher order oligomers of the polymerase heterotrimer can be detected in highly purified preparations of virus RNA polymerase and discuss the possible implications of this observation.

To express the recombinant virus polymerase, cultures of HEK 293T cells were infected with vTF7-3 virus and subsequently transfected with plasmids pGPF1TAP (or pGPB1 as a control), pGPB2 and pGPA (Fig. 1a), using a calcium phosphate transfection protocol. Total cell soluble extracts were applied to IgG–Sepharose beads (Amersham Pharmacia), the resin was washed extensively and the polymerase was eluted by digestion with tobacco etch virus.
After extensive washing, the proteins that bound calmodulin–agarose beads in the presence of calcium. The eluates were pooled and applied to gel-filtration analysis of purified influenza polymerase-ferritin; CT, catalase; BSA, BSA.

**Fig. 1.** Gel-filtration analysis of purified influenza polymerase-containing complexes. Wild-type polymerase polPB1TAP was expressed and purified by the TAP approach as described previously (Villacé et al., 2004). (a) Diagram of the polymerase complex with a TAP tag attached at the C terminus of PB1 subunit. The TAP tag (Rigaut et al., 1999) allows the purification of protein complexes under very mild conditions by two steps of affinity chromatography, IgG–Sepharose and calmodulin–agarose. (b) Characterization of the purified material (PB1TAP) by Western-blot using anti-PB1, anti-PB2 and anti-PA antibodies. The analysis of a control (CTRL), purification of untagged polymerase complex, is also shown. (c) Protein composition of the purified material as determined by silver staining. The position of the polymerase subunits (PB1CBD, PB2/PA) is marked with arrowheads and determined by silver staining. The position of the polymerase (Fig. 1b) and was highly purified. The polymerase subunits were clearly detectable in the sample (Fig. 1c, arrowheads), although host-cell-associated factors were also detected, as described elsewhere (N. Jorba and others, unpublished data). The size of the purified polymerase complexes was analysed by gel filtration over a calibrated Sephacryl S400 column and the results are presented in Fig. 1(d). Most of the polymerase, revealed by Western-blot using anti-PA antibodies, co-migrated with the catalase marker (MW 220 kDa), as expected for the heterotrimeric form of the enzyme. Similar results were obtained when the polymerase was revealed with antibodies specific for the PB2 subunit (data not shown). As these complexes were purified by affinity chromatography with tandem affinity purification (TAP)-tagged PB1, the results show that essentially heterotrimeric polymerase was formed and that PB1–PB2 and PB1–PA heterodimers, which could potentially be formed in the transfected cells, were not apparent in the purified samples (Fig. 1c and data not shown). On the contrary, the polymerase signal was skewed towards higher molecular mass forms and showed that some of the polymerase co-migrated with the ferritin and thyroglobulin markers (440 and 670 kDa, respectively). In addition, some purified polymerase appeared close to the column exclusion marker, in the MDa range. These results suggest that oligomeric forms of the polymerase complex are formed in vivo.

To explore the possibility of a polymerase heterotrimer–heterotrimer interaction in vivo, the polymerase was purified after expression of its subunits, one of which contained alternative tags in the same position. We used PB1TAP/PB1, PB2TAP/PB2His and PATAP/PA as alternatively tagged subunits in independent expression experiments. This approach would lead to the formation of two alternative heterotrimers in each experiment, namely PB1TAP–PB2–PA and PB1–PB2–PA or PB1–PB2TAP–PA and PB1–PB2His–PA or PB1–PB2–PATAP and PB1–PB2–PA, respectively (Fig. 2a, left). If both heterotrimers interact in vivo, purification by the TAP procedure should lead to the co-purification of the untagged PB1, the PB2His marker or the untagged PA, respectively. As controls, the wild type (wt) polymerase was expressed by itself or co-expressed with the PB2His subunit and purified in parallel by the TAP method (Fig. 2a, right). The results obtained for PB1TAP–PB2–PA/PB1–PB2–PA, for PB1–PB2TAP–PA/PB1–PB2His–PA and for PB1–PB2–PATAP/PB1–PB2–PA are presented in Fig. 2(b, c and d), respectively. Western-blot analyses are shown for the purified material with antibodies specific for (i) PB1, PB2 or PA, that would reveal all versions of these subunits; (ii) His tag, that would reveal PB2His and (iii) CBD tag, that would show the PB1, PB2 or PA protein resulting from the TAP-tagged subunit after TAP purification. In agreement with the results presented in Fig. 1, no signal was detected in control purifications with any of the antibodies used (Fig. 2b, c and

protease. The eluates were pooled and applied to calmodulin–agarose beads in the presence of calcium. After extensive washing, the proteins that bound specifically were eluted with an EGTA-containing buffer. The material thus obtained contained all three subunits of the polymerase (Fig. 1b) and was highly purified. The polymerase subunits were clearly detectable in the sample (Fig. 1c, arrowheads), although host-cell-associated factors were also detected, as described elsewhere (N. Jorba and others, unpublished data). The size of the purified polymerase complexes was analysed by gel filtration over a calibrated Sephacryl S400 column and the results are presented in Fig. 1(d). Most of the polymerase, revealed by Western-blot using anti-PA antibodies, co-migrated with the catalase marker (MW 220 kDa), as expected for the heterotrimeric form of the enzyme. Similar results were obtained when the polymerase was revealed with antibodies specific for the PB2 subunit (data not shown). As these complexes were purified by affinity chromatography with tandem affinity purification (TAP)-tagged PB1, the results show that essentially heterotrimeric polymerase was formed and that PB1–PB2 and PB1–PA heterodimers, which could potentially be formed in the transfected cells, were not apparent in the purified samples (Fig. 1c and data not shown). On the contrary, the polymerase signal was skewed towards higher molecular mass forms and showed that some of the polymerase co-migrated with the ferritin and thyroglobulin markers (440 and 670 kDa, respectively). In addition, some purified polymerase appeared close to the column exclusion marker, in the MDa range. These results suggest that oligomeric forms of the polymerase complex are formed in vivo.

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d, right). On the contrary, a clear band of reactivity with anti-His antibody was observed in the TAP-purified material after co-expression of PB2TAP- and PB2His-containing polymerase (Fig. 2c, left, His). This was consistent with the appearance of a doublet in the signal with anti-PB2 antibody (Fig. 2c, left, PB2), one of which co-migrated with the signal detected with the anti-CBD antibody (Fig. 2c, left, CBD) and the other with the anti-His antibody. Finally, the appearance of PA-specific signal (Fig. 2c, left, PA) verified that a heterotrimer had been purified during the TAP procedure. Similar results were obtained for the co-expression of polymerase with tagged and untagged PB1 or PA subunits (Fig. 2b and d). Thus, the reactivity with anti-PB1 or anti-PA antibody of the TAP-purified material after co-expression of PB1TAP or PATAP and wt polymerase showed a doublet signal (Fig. 2b, left, PB1; Fig. 2d, left, PA), the stronger of which co-migrated with the signal observed with the anti-CBD antibody (Fig. 2b, left, CBD; Fig. 2d, left, CBD). Verification of heterotrimer formation was carried out by Western-blot using antibodies specific for PB2 (Fig. 2b, left, PB2; Fig. 2d, left, PB2). To exclude the possibility that the double bands observed for PB1 or PA in Fig. 2(b) or Fig. 2(d), respectively, are the consequence of protein proteolysis, similar purifications were performed in the absence of untagged subunit expression. The results indicated that only the PB1CBD or PACBD were detected and no double band was observed (see Supplementary Fig. S1 available in JGV Online).

Taken together, the results presented in Figs 1 and 2 indicate that the influenza virus polymerase heterotrimer can form oligomers in vivo. Thus, the gel-filtration pattern presented in Fig. 1 shows polymerase aggregates with apparent molecular masses of 440, 670 kDa and higher. On the other hand, the TAP co-purification experiments
indicate the association of polymerase complexes irrespective of the subunit that was differentially tagged. However, these results do not determine which subunit may participate in the associations among polymerase heterotrimers. To analyse this question only PB1TAP/PB1, PB2TAP/PB2His or PATAP/PA subunits were co-expressed instead of complete polymerase complexes (Fig. 3a). After purification by the TAP procedure, the purified material was probed with antibodies specific for the particular polymerase subunit expressed and the presence of the tagged subunit was verified by Western-blot with the CBD tag (data not shown). Co-purification was detected for untagged PB1 or PB2His with PB1TAP or PB2TAP, respectively (Fig. 3b, c, left). However, no interaction was observed between PATAP and PA subunits (Fig. 3d, left). These results are in agreement with previous data (Digard et al., 1989) and suggest that the PB1 and/or PB2 subunits might be involved in the polymerase–polymerase association, although we cannot conclude a direct interaction between PB1 and/or PB2 since we cannot exclude the participation of a host factor(s) in the oligomerization in vivo.

At present we cannot verify the existence of the polymerase oligomeric forms in virus-infected cells but, if this were the case, these polymerase associations might be important for RNA transcription and/or replication in the infection cycle, as has been previously reported for positive-stranded RNA viruses, like poliovirus or hepatitis C virus (Lyle et al., 2002; Wang et al., 2002) and negative-stranded RNA viruses, like Sendai virus (Cevik et al., 2003; Smallwood et al., 2002). In the influenza RNA replication process, the first nascent virus RNA contains the 5'--terminal conserved sequence, which is an efficient and specific binding site for the polymerase (González & Ortín, 1999; Lee et al., 2002; Tiley et al., 1994). This recognition might be facilitated by an association of the incoming polymerase complex to the RNP-resident polymerase. As for many other viruses, oligomerization of the influenza virus replication complexes might provide important advantages for the efficiency of the interactions among virus and host factors required for RNA replication and transcription (reviewed by Ortín & Parra, 2006). However, an additional possibility could be considered for the requirement of polymerase–polymerase interactions in vivo. Thus, the process of encapsidation of the segmented genome into virus particles might require multiple contacts between the polymerase complexes present in the virion RNPs and, indeed, a mutation in the PA subunit has been described that is defective in particle formation (Regan et al., 2006).

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References


