A conserved arginine residue in the terminal protein domain of hepatitis B virus polymerase is critical for RNA pre-genome encapsidation

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Hepadnaviruses, including human hepatitis B virus (HBV) and duck hepatitis B virus (DHBV), replicate their DNA genome through reverse transcription. Although hepadnaviral polymerase (Pol) is distantly related to retroviral reverse transcriptases, some of its features are distinct. In particular, in addition to the reverse transcriptase and RNase H domains, which are commonly encoded by retroviral reverse transcriptases, the N-terminally extended terminal protein (TP) domain confers unique features such as protein-priming capability. Importantly, the TP domain is also essential for encapsidation of the viral RNA pre-genome. To gain further insight into the TP domain, this study used clustered charged residue-to-alanine mutagenesis of HBV Pol. Of the 20 charged residues examined, only one arginine (R105) was critical for RNA encapsidation. This result contrasts with previous findings for DHBV Pol regarding the critical residue of the TP domain required for RNA binding. Firstly, R128 of DHBV Pol, which corresponds to R105 of HBV Pol, was reportedly tolerable to alanine substitution for RNA binding. Secondly, the C-terminal arginine residue of the DHBV Pol TP domain (R183) was shown to be critical for RNA binding, whereas alanine substitution of the corresponding arginine residue of the HBV Pol TP domain (R160) remained able to support RNA encapsidation. Together, these data highlight the divergence between avian and mammalian hepadnaviral Pols with respect to an arginine residue critical for RNA encapsidation.

INTRODUCTION

Hepatitis B virus (HBV) infection represents a major global public health problem, with over 360 million chronically infected patients worldwide (Shepard et al., 2006). Chronic HBV infection is associated with a substantially higher risk of developing severe liver diseases, including cirrhosis and hepatocellular carcinoma, which results in approximately 1 million deaths annually. Nucleoside analogues such as lamivudine and adefovir are used to treat patients with chronic HBV infections, but the utility of these treatments is limited as a result of the emergence of drug-resistant variants (Zoulim & Locarnini, 2009), indicating a clear medical need for new therapeutic strategies. HBV is the prototypic member for the family Hepadnaviridae, which also includes duck hepatitis B virus (DHBV) and the woodchuck hepatitis virus (WHV). The genetic organization of all members of the family is highly conserved (Seeger et al., 2007). Each virus has a small (~3.2 kb) circular DNA genome that encodes the nucleocapsid (core), the polymerase (Pol) and the envelope (surface antigen), together with the X gene, in a compact, overlapping arrangement.

Although HBV contains a DNA genome, it replicates via reverse transcription of an RNA template, the pre-genomic RNA (pgRNA) (Beck & Nassal, 2007). This replication strategy underlines HBV’s relatedness to retroviruses and, as such, hepadnaviral Pol shares many features with retroviral reverse transcriptases (Nassal, 2008). In addition to the reverse transcriptase and RNase H domains, which are commonly encoded by retroviral reverse transcriptases, two domains are encoded at the N terminus of hepadnaviral Pols: the terminal protein (TP) and spacer (SP) domains (Fig. 1) (Radziwill et al., 1990). The SP domain was initially thought to serve merely as a tether that links the TP and reverse transcriptase domains, because much of it can be deleted without affecting the functions of Pol (Hu et al., 2004; Radziwill et al., 1990). However, we recently demonstrated that the C-terminal one-quarter of the SP domain is indispensable for RNA binding (Kim et al., 2009). Compared with the SP domain, which is highly divergent, the TP domain is well conserved among hepadnaviral Pols. In particular, the TP domain contains an invariant tyrosine residue (Y63 in HBV and Y96 in DHBV; see Supplementary Fig. S1, available in JGV Online).
that primes DNA synthesis and covalently links hepadnaviral Pols to the viral DNA by a reaction called protein priming (Radziwill et al., 1990; Wang & Seeger, 1992). Thus, the TP domain itself is covalently linked to the viral DNA and serves as a protein primer for DNA synthesis.

In addition to the invariant tyrosine residue, the C-terminal region of the TP domain, often referred to as the T3 motif, has been shown to contribute to protein priming. In fact, the T3 motif was first identified as a highly conserved segment by alignment of the C-terminal one-third of the TP domain (Cao et al., 2005). Multiple in vitro studies with DHBV Pol have indicated that the charged residues in the T3 motif participate directly in RNA binding in a manner dependent on cellular chaperones such as heat-shock protein 70 (Hsp70). Specifically, a double mutation of two charged residues within the T3 motif (K182/R183) has been shown to block protein priming and RNA binding, underlining the importance of these positively charged residues for the recognition of the viral RNA (Badtke et al., 2009; Cao et al., 2005; Seeger et al., 1996). Likewise, residues K161/R162 in the HBV subtype adw2 Pol, which correspond to K182/R183 of DHBV, have been reported to be essential for viral genome replication (Cao et al., 2005), implying conservation of T3 motif functions among hepadnaviruses. Overall, two distinct regions of the TP domain have been shown to participate in the protein priming reaction: (i) the invariant tyrosine residue, which primes DNA synthesis and (ii) the C-terminal region, which is essential for RNA binding.

To gain further insight into the contribution of the TP domain to viral reverse transcription, we carried out extensive clustered CA mutagenesis spanning the entire TP domain. In contrast to the expectations from previous studies carried out with DHBV Pol, alanine substitution of the conserved charged residues in the C-terminal region of the HBV Pol TP domain were well tolerated with regard to RNA encapsidation. Instead, our results revealed that R105 of the HBV Pol TP domain is critical for RNA encapsidation. Together, these data reveal the divergence between avian and mammalian hepadnaviral Pols. Nevertheless, we do not exclude the possibility that non-clustered charged residues not examined in this work could contribute to pgRNA encapsidation, in addition to R105.

RESULTS AND DISCUSSION

CA mutagenesis

Clustered CA mutagenesis has been used successfully to examine the contribution of charged amino acid residues to a specific protein function (Diamond & Kirkegaard, 1994; Seeger et al., 1996). In this study, mutants were designed such that two of the following four charged amino acids (arginine, lysine, aspartic acid and glutamic acid) within a span of no more than 5 aa were changed to alanine. Clusters of charged amino acids, typically located on the surface of proteins, are more likely to participate in specific interactions between the protein and other molecules. Thus, substituting these residues with alanine causes minimal alterations to the polypeptide but could disrupt specific interactions (Alber, 1989). To gain structural insight into the TP domain, we sought to identify specific charged residues within the TP domain that contribute to viral reverse transcription. Ten CA mutants were generated, spanning the entire TP domain (Fig. 1), and the mutants were examined for their ability to support viral genome replication and RNA pre-genome encapsidation.

Identification of a CA mutant defective in viral genome replication and RNA encapsidation

We employed a complementation strategy to examine the ability of the HBV Pol mutants to support viral DNA synthesis, as described previously (Kim et al., 2009). HepG2 cells were transfected with each CA mutant along with an HBV 1.3mer replicon construct that yields the pgRNA but encodes a frame-shift mutation in the P ORF that abolishes Pol expression (HBV 1.3mer P-null; Wang et al., 2009). At 4 days post-transfection, cytoplasmic capsids were isolated, the viral DNA replication intermediates were extracted from the isolated capsids and the replication intermediates were analysed by Southern blot analysis. Southern blot analysis confirmed that the wild-type (WT) Pol supported the synthesis of three DNA replication intermediates: ssDNA, duplex linear DNA and relaxed circular DNA (Fig. 2a, lane 1). Unexpectedly, all of
the HBV Pol mutants, except for CA8, supported viral genome replication at levels comparable to WT Pol (Fig. 2a). The data indicated that most of the charged residues (18/20 residues) were not critical for viral genome replication. The CA8 mutant corresponded to mutation of K104 and R105 and indicated that one or both of these residues is essential for viral genome replication.

To identify the step at which the CA8 mutant was defective in viral genome replication, we measured encapsidation of pgRNA, a step prior to viral DNA synthesis. Cells were transfected as above and, at 3 days post-transfection, total RNA and capsid-associated RNA was extracted and analysed by RNase protection analysis (RPA; Fig. 2b). Consistent with the data above, RPA revealed that HBV Pol derived from the CA8 mutant failed to support pgRNA encapsidation (Fig. 2b, lower panel, lanes 7 and 8), indicating that the defect in viral genome replication occurred at the step of viral RNA encapsidation.

The R105 residue of HBV Pol is critical for RNA encapsidation

To determine whether K104 or R105 was the single residue critical for RNA encapsidation, these two residues were mutated individually to alanine (K104A and R105A mutants). HepG2 cells were transfected and Southern blot analysis of virus replication intermediates was performed, as described above. The results showed that HBV Pol derived from the K104A mutant supported viral genome replication to a level similar to that of WT HBV Pol, but the R105A mutant failed to support genome replication, indicating that the R105 residue is critical for viral genome replication (Fig. 3a, lanes 3 and 4). RPA performed in parallel confirmed that the R105A mutant also failed to encapsidate pgRNA (Fig. 3b, lanes 5 and 7). Western blot analysis confirmed that HBV Pol derived from the CA8 mutant was expressed at levels similar to WT Pol (Fig. 3c, lane 3), but that both the K104A and R105A mutants were expressed at slightly reduced levels compared with WT Pol (Fig. 3c, compare lane 2 with lanes 4 and 5). As both of

![Fig. 2. Analysis of CA mutants for their ability to support viral genome replication. (a) HepG2 cells were co-transfected with the HBV P-null construct along with the WT or mutant Pol expression constructs as indicated. Southern blot analysis was performed to detect the three HBV replication intermediates: relaxed circular DNA (RC), duplex linear DNA (DL) and ssDNA (SS). A restriction fragment representing one HBV genomic unit of 3.2 kb served as a size marker (SM). The assay was performed in triplicate and a representative Southern blot is shown. The percentage of virus replication is expressed relative to WT Pol (means ± SD). (b) RPA to measure the efficiency of pgRNA encapsidation. HepG2 cells were transfected with the CA mutant plasmids along with the HBV P-null construct. Three days after transfection, total RNA (T) and capsid-associated RNA (C) were extracted and analysed by RPA. pgRNA is denoted by a filled arrowhead, whilst the riboprobe is denoted by an open arrowhead. Yeast RNA was used as a negative control. The value for the WT was set at 100%. Results are shown as means ± SD from three independent experiments.

![Fig. 3. Only residue R105 is critical for viral DNA synthesis and encapsidation. (a) Southern blot analysis was performed as described in Fig. 2(a). (b) RPA was performed as described in Fig. 2(b). (c) Western blot analysis of HBV Pol was performed using an anti-Flag antibody to demonstrate equal Pol expression levels. β-Actin served as a loading control.](http://vir.sgmjournals.org)
the K104A and R105A mutants expressed HBV Pol to similar relative levels, the slight reduction relative to WT HBV Pol cannot account for the dramatic reduction in RNA encapsidation observed. Based on the above results, we concluded that the R105 residue of the HBV Pol TP domain is indispensable for RNA encapsidation.

To gain further insight into the role of the R105 residue in RNA encapsidation, we sought to assess the in vivo RNA-binding activity of the R105A HBV Pol mutant. As direct RNA binding by HBV Pol is activated only following in vitro reconstitution of the Hsp90 chaperone complex (Hu et al., 2004), we instead employed a surrogate RNA-binding assay that essentially measures the ability of a co-transfected competitor (Pol mutants) to inhibit WT Pol-driven viral DNA synthesis (Kim et al., 2009). This indirect RNA-binding assay exploits the Y63F mutant of HBV Pol, which is defective in protein-priming activity but retains RNA-binding activity. The inhibitory effect of the Y63F mutant Pol on viral DNA synthesis would be more pronounced with a high mutant-to-WT Pol ratio. Thus, cells were co-transfected with 1 : 3 ratios of WT versus mutant plasmid, along with the HBV P-null construct. Capsid-associated viral DNA replication intermediates were then analysed by Southern blot analysis. As reported previously (Kim et al., 2009), co-transfection of the Y63F mutant plasmid substantially reduced the level of viral genome replication of WT Pol (Fig. 4a, compare lanes 1 and 2). In contrast, co-transfection of the Y63F/R105A double mutant did not significantly diminish the level of viral DNA synthesis driven by WT Pol (Fig. 4a, lane 3), although the expression levels of the Y63F and Y63F/R105A HBV Pol protein were similar by Western blot analysis (Fig. 4b). These data were interpreted as indicating that the Y63F/R105A mutant failed to inhibit WT Pol DNA synthesis due to a lack of RNA binding caused by the R105A mutation.

HBV Pol R105A mutant remains able to interact with HBV core and Hsp90 proteins

HBV Pol is known to interact with viral proteins as well as cellular proteins, including the viral core protein and Hsp90 (Hu et al., 2004; Lott et al., 2000). Hence, another possibility to explain why the R105A mutant is defective for RNA encapsidation is that HBV Pol derived from the R105A mutant could fail to interact with the viral core proteins. Co-immunoprecipitation analysis showed that HBV Pol derived from the R105A mutant remained able to interact with the core protein at a level comparable to WT Pol (Fig. 5a, compare lanes 2 and 3), indicating that the R105 residue is unimportant for the interaction with the viral core protein. Nevertheless, it should be noted that the extent to which the direct interaction between HBV Pol and the core protein is relevant to pgRNA packaging is unclear, given that neither HBV Pol or pgRNA alone can be packaged in the absence of each other (Bartenschlager & Schaller, 1992; Pollack & Ganem, 1994).

Next, we assessed the possibility that the R105A mutant could fail to interact with Hsp90. As shown in Fig. 5(b), co-immunoprecipitation data showed that HBV Pol from the R105A mutant remained able to interact with Hsp90 (Fig. 5b, lane 3). Altogether, the data indicated that the packaging defect of the R105A mutant was not attributed to a defect in its interactions with the viral core protein and cellular Hsp90. A minor possibility that the defect in the R105A mutant in RNA encapsidation could be due to subcellular mislocalization was excluded, as both WT and R105A HBV Pol were detected predominantly in the cytoplasm and their distribution patterns were indistinguishable from each other by indirect immunofluorescence (data not shown). Overall, the data shown in Figs 3–5 support the interpretation that the R105 residue is critical for pgRNA encapsidation, perhaps acting at the level of RNA binding.

Divergence between mammalian and avian hepadnaviral Pols

The preceding data showed that R105 alone is critical for pgRNA encapsidation (Figs 3–5). In contrast, it has been reported that a double substitution of the two charged residues in the C-terminal region of the TP domain of HBV Pol (K161E/R162E in subtype adw2; Fig. 6a), completely eliminates viral genome replication (Cao et al., 2005). Furthermore, the corresponding R183 of DHBV Pol has been shown to be critical for RNA binding in vitro.
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(Stahl et al., 2007). In contrast, our data showed that the corresponding double substitution of K159/R160 in subtype ayw (the CA10 mutant) did not affect viral DNA synthesis (Fig. 2a, lane 11; Fig. 2b, lower panel, lanes 11 and 12). In addition, the HBV Pol R160A mutant was proficient for viral genome replication and pgRNA encapsidation (Fig. 3a, lane 5 and Fig. 3b, lane 9). Furthermore, the R160A mutant competed fully with WT HBV Pol for viral DNA synthesis in an RNA-binding competition assay (Fig. 4a, lane 4). Overall, these data indicate that residue R160 is dispensable for RNA binding. Therefore, a discrepancy clearly exists regarding the importance of the conserved arginine residue in the C-terminal region of the TP domain, which is often referred to as the T3 motif (Fig. 6a; Cao et al., 2005).

One possible explanation for the discrepancy is that the mutation examined by Cao et al. (2005) reversed the residue's charge from the positively charged lysine or arginine (K or R) to the negatively charged glutamic acid (E). Thus, we reasoned that the defect of the K161E/R162E mutants could be due to the drastic change of amino acid residue. To assess this possibility, a K159E/R160E substitution mutant of HBV Pol subtype ayw, which corresponds to the K161E/R162E substitution of the subtype adw2 (Fig. 6a), was generated. In addition, the individual single amino acid substitution mutants K159E and R160E were also produced. In good agreement with the previous report (Cao et al., 2005), the K159E/R160E mutant failed to support viral DNA synthesis (Fig. 6b, lane 2). Further analysis of the two single mutants revealed that the R160E mutation was, in fact, responsible for the defect (Fig. 6b, lane 4). RPA performed in parallel showed that the R160E HBV Pol mutant was defective in pgRNA encapsidation (Fig. 6c). Western blot analysis confirmed comparable expression of the mutant and WT Pol proteins (Fig. 6d). Thus, these data showed that residue R160 (subtype ayw) per se is not critical for RNA encapsidation but that a particular mutation of this residue could abolish the replication activity.

Based on the above data (Figs 3–6), we concluded that the conserved arginine residue in the C-terminal TP domain (R160 in subtype ayw or R162 in subtype adw2) is dispensable for viral genome replication and RNA encapsidation. This observation highlights the divergence between mammalian and avian hepadnaviral Pols with respect to the TP domain. Overall, although the R183 residue of DHBV Pol was critical for RNA binding, the corresponding R160 residue of HBV Pol was dispensable for RNA encapsidation (Fig. 6a).

The genetic analysis presented here has uncovered important disparities between avian and mammalian hepadnaviral Pols with respect to the charged residue essential for RNA encapsidation. Firstly, we showed that residue R105 of HBV Pol is critical for pgRNA encapsidation (Figs 2 and 3). By contrast, the corresponding R128 residue of DHBV Pol was critical for RNA binding, the corresponding R160 residue of HBV Pol was dispensable for RNA encapsidation (Fig. 6a).

The genetic analysis presented here has uncovered important disparities between avian and mammalian hepadnaviral Pols with respect to the charged residue essential for RNA encapsidation. Firstly, we showed that residue R105 of HBV Pol is critical for pgRNA encapsidation (Figs 2 and 3). By contrast, the corresponding R128 residue of DHBV Pol was critical for RNA binding, the corresponding R160 residue of HBV Pol was dispensable for RNA encapsidation (Fig. 6a).

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inferred that the R128 residue of DHBV Pol is dispensable for RNA binding. To be certain that the disparity observed is not caused by the particular double mutations, single substitution mutagenesis of the R128 residue of DHBV Pol is needed. Moreover, the observation that these two DHBV Pol mutants (R128A plus K131A, and E124A plus R128A) were modestly defective in RNA packaging (14 and 20% of WT level, respectively) but more severely defective in DNA replication has suggested that the R128 residue of DHBV Pol could contribute to either capsid assembly or minus-strand DNA synthesis (Seeger et al., 1996). In addition, to evaluate the importance of the highly conserved arginine residue among members of the family Hepadnaviridae, parallel genetic analysis of WHV and woolly monkey hepatitis B virus Pol merits further investigation (see Supplementary Fig. S1).

Secondly, an earlier report on the importance of the C-terminal region of the TP domain of HBV Pol (Cao et al., 2005) was disputed here by parallel substitution analysis (Fig. 6), revealing that R160 (subtype ayw) per se is dispensable for RNA encapsidation. It could be that a drastic alteration such as R160E substitution may lead to misfolding of the HBV Pol polypeptide (Fig. 6b, lane 4). Overall, we showed that residue R160 of HBV Pol is dispensable for RNA encapsidation (Figs 3 and 6). By contrast, the corresponding R183 residue of DHBV Pol has been shown to be critical for RNA binding in vitro (Stahl et al., 2007), revealing a divergence between avian and mammalian hepadnaviral Pols.

Cellular chaperones are required for the RNA-binding activity of both HBV and DHBV Pol (Hu et al., 2004). In the case of DHBV Pol, it has been shown that chaperones, including Hsp70 and its co-chaperone, Hsp40, facilitate partial refolding of the C-terminal region in the TP domain such that this region becomes exposed and accessible for viral RNA binding (Cao et al., 2005; Stahl et al., 2007). The work presented here has shown that residue R105, which is somewhat distantly located from the C-terminal region of the TP domain, is critical for RNA encapsidation, most probably at the level of RNA binding. As the RNA-binding capacity of HBV Pol is conferred by cellular chaperones as well (Hu et al., 2004), it is possible that, like DHBV, chaperone-mediated refolding of HBV Pol makes the region around R105 accessible for RNA binding. Further investigation is needed to address this hypothesis.

The TP domain of hepadnaviral Pol is unique among viral reverse transcriptase proteins, making it an attractive target for therapeutic intervention (Nassal, 2009). However, at present, no structural information is available for the TP domain. In the absence of an atomic structure, genetic analyses such as that demonstrated here are instrumental for a deeper understanding of viral reverse transcription, which may lead to the development of more effective antiviral drugs for the treatment of chronic HBV infection.

**METHODS**

**Cell culture and transfection.** HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco-BRL) and 10 μg gentamicin ml⁻¹ at 37 °C in 5% CO₂ and were passaged every third day. Cells were transfected using polyethyleneimine (25 kDa; Sigma-Aldrich), as described previously.

![Fig. 6. A conserved arginine residue in the T3 motif of the TP domain is dispensable for RNA binding.](image-url)
Plasmid construction. The HBV P-null replicon construct has been described previously (Ryu et al., 2008). It was made by introducing two mutations into the HBV replicon: (i) a frame-shift mutation by deletion of the T nucleotide of the second ATG of the P ORF and (ii) a point mutation that changed the first ATG of the P ORF into ACG without altering the amino acids encoded by the overlapping C ORF (Ryu et al., 2008). PCMV–Pol is an HBV polymerase expression construct, described previously (Ryu et al., 2008), which was used to produce HBV Pol with three copies of the Flag tag at the N terminus. All mutants were sequenced to confirm the base changes.

Southern blot analysis. Viral DNAs from cytoplasmic capsids were isolated from HepG2 cells 4 days after transfection as described by Nassal (1992) and analysed by Southern blotting, performed as described previously (Shin et al., 2004) to measure the viral replication intermediates. Briefly, extracted viral DNA was separated by electrophoresis through a 1.3 % agarose gel in a 0.5 x Tris/acetate/EDTA buffer and transferred onto a nylon membrane. The nylon membrane was pre-hybridized with hybridization solution and then hybridized with a 32P-labelled full-length HBV DNA probe in hybridization solution for 16 h at 65 °C. Images were obtained using a phosphomager (BAS-2500; Fujiﬁlm).

Western blot analysis. Western blot analysis was performed essentially as described previously (Cha et al., 2009). Protein samples were resolved by SDS-PAGE (10 % polyacrylamide) and then transferred to a PVDF membrane (Immobilon-P; Millipore). After blocking, the membrane was incubated with a mouse anti-Flag M2 antibody (diluted 1 : 5000; Amersham), respectively. β-Actin was detected using a rabbit anti-β-actin antibody (diluted 1:5000; Sigma). Proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer). Images were quantified using LAS-3000 (Fujiﬁlm).

RNA extraction and RNase protection analysis. RNA from whole cells and cytoplasmic capsids was extracted 3 days after transfection, as described previously (Cha et al., 2009). Briefly, HepG2 cells were washed twice with cold HBSS/EGTA buffer [2 mM HEPES (pH 7.5), 100 mM NaCl, 0.5 mM EGTA] and lysed in lysis buffer [50 mM Tris/ HCl (pH 8.0), 1 mM EDTA, 1 % NP-40, 100 mM NaCl]. Capsids were collected by immunoprecipitation using anti-core antibody (Dako). Briefly, cell lysate was mixed with anti-core antibody for 1 h at 4 °C. Protein–Sepharose was added and the mixture was incubated for an additional 2 h at 4 °C. Samples were washed with lysis buffer and immunoprecipitates were collected by centrifugation. Total and capsid-associated RNA was extracted with Trizol (Gibco-BRL). RNA was analysed by RNase protection analysis, according to the manufacturer’s protocol (Ambion). The riboprobe was derived from the core region (nt 1903–2140), as described elsewhere (Ryu et al., 2008). Briefly, each sample of RNA was hybridized with a probe labelled with 32P c.p.m. [α-32P]UTP (3000 Ci mmol−1; Amersham) for 16 h at 42 °C. RNA digestion was carried out with a mixture of RNase A and RNase T1 for 30 min at 37 °C. The digested products were separated on 5 % acrylamide/8 M urea gels. Images were obtained using a phosphomager.

Co-immunoprecipitation. Immunoprecipitation was performed essentially as described elsewhere (Wang et al., 2009). After transient transfection, the medium was removed and the cells were rinsed twice in cold PBS, incubated for 30 min at 4 °C in lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 % NP-40] and collected by scraping. Cell debris was removed by centrifugation at 10 000 g for 10 min at 4 °C. Extracts were pre-cleared with protein G-agarose beads for 1 h at 4 °C. The primary antibody was added for 1 h at 4 °C, and immunoglobulin complexes were collected on protein G–agarose beads for 1 h at 4 °C. The beads were washed five times with 1 ml lysis buffer each. Protein complexes were generally recovered by boiling in Laemmli sample buffer and were analysed by SDS-PAGE.

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