Rabies virus chaperone: Identification of the phosphoprotein peptide that keeps nucleoprotein soluble and free from non-specific RNA

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Abstract

The genomic RNA of rabies virus is always complexed with the viral nucleoprotein (N). This N–RNA complex is the template for viral transcription and replication. The viral phosphoprotein (P) has two functions during the infection process: it binds through its carboxy-terminus to N in the N–RNA complex and at the same time with an amino-terminal domain to the polymerase and in this way fixes the polymerase to its template. The second function of P is to bind to newly produced N in the infected cell in order to prevent that N binds non-specifically and irreversibly to cellular RNA. In order to identify the part of the phosphoprotein that binds to N and keeps the latter soluble, we isolated the N–P complex, performed sequential protease digestions, and determined the identity of the remaining N and P peptides in the purified digested complex. Although the digestion steps removed short sequences of N, most of N remained intact and soluble, indicating that the overall structure was not affected. Most of P, including the carboxy-terminal N–RNA-binding domain, was removed during the first digestion step. N-terminal sequencing and mass spectrometry analysis identified a P peptide containing residues 4–40 that remained associated with N. Coexpression and coimmunoprecipitation experiments and yeast two-hybrid experiments showed that this peptide alone could bind to N in vivo.

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Introduction

Non-segmented negative strand RNA (NNSR) viruses (rabies, measles, respiratory syncytial, or Ebola viruses) have a genome that is always associated with the viral nucleoprotein (N). This N–RNA complex is the template for transcription and replication rather than the naked viral RNA (Arnheiter et al., 1985). When recombinant N is expressed in bacteria or eukaryotic cells, it binds to cellular RNA and forms N–RNA complexes with the same morphology and N:RNA stoichiometry as the viral N–RNA (Spehner et al., 1991; Fooks et al., 1995; Iseni et al., 1998). However, in infected cells N binds predominantly to newly produced viral RNA, not to cellular RNA. The reason for this difference is the presence of a viral chaperone protein, the phosphoprotein (P), which binds to newly formed N and prevents it from binding to non-viral RNA (Masters and Banerjee, 1988). Such rabies virus N–P complexes, consisting of one copy of N and two copies of P, were isolated and characterized from insect cells coexpressing recombinant N and P proteins (Mavrakis et al., 2003).

P has a second crucial role in the infection process as polymerase cofactor. An oligomeric form of P binds to the nucleoprotein in the N–RNA and at the same time to the
viral polymerase in such a way that it positions the polymerase onto its template (Mellon and Emerson, 1978). Possibly related to these two independent functions of P is the fact that the molecule has two sites for binding to N (Fu et al., 1994; Chenik et al., 1994; Jacob et al., 2001). Binding of P to the carboxy-terminal domain of N in N–RNA (Schoehn et al., 2001) is mediated through a carboxy-terminal domain of P (residues 186–296), the X-ray structure of which was recently determined (Mavrakis et al., 2004). The limits of the second N-binding domain of P have not yet been clearly identified except that it is situated on the amino-terminal half of P (Fu et al., 1994; Chenik et al., 1994; Jacob et al., 2001). Sendai virus is an NNSR virus from a different family (Paramyxoviridae) than rabies virus (Rhabdoviridae). Sendai virus P has also two domains that bind to N, a carboxy-terminal domain binding N–RNA (Longhi et al., 2003; Blanchard et al., 2004; Kingston et al., 2004) and a domain that binds and chaperones N in a soluble N–P complex. This second domain is situated close to the amino-terminus of Sendai virus P (residues 33–41) (Curran et al., 1995) and is predicted to form a helical secondary structure. Because a conserved domain at the amino-terminus of rabies virus P is also predicted to form a helical structure, it was proposed that it is this domain that binds to N in the N–P complex (Mavrakis et al., 2004).

In this paper, we performed limited proteolysis on purified rabies virus N–P complex. Most of the phosphoprotein was removed during these digestions; however, most of the nucleoprotein remained intact. By N-terminal protein sequencing and mass spectrometry, we could identify peptides corresponding to residues 4–40 of P that remained associated with N after extensive digestion. Through coexpression and coimmunoprecipitation and yeast two-hybrid experiments, we show that this peptide alone binds to N in the absence of the rest of the phosphoprotein.

**Results**

Soluble rabies virus N–P complex was produced in insect cells and purified as described (Mavrakis et al., 2003). The N–P complex was digested with trypsin, and after quenching the digestion reaction, the sample was loaded onto a Superdex-75 size exclusion chromatography column. The elution profile with two peaks containing nucleic acid-free protein and the Tris–tricine gels of the eluate are shown in Fig. 1. For all proteins visualized by Coomassie blue staining, we performed N-terminal sequencing. Peak I contained only fragments of the nucleoprotein N. The bands corresponded to sequences starting from amino acid (aa) 10, aa 359, aa 377, and aa 401 of the nucleoprotein (Fig. 1b). When in a second experiment, the sample was more extensively digested, the band starting with aa 359 disappeared (Fig. 1c). We did not perform mass spectrometry analysis on the protein in these bands, but based on the apparent molecular weights, the bands most likely correspond to aa 10–358 (39 kDa), 359–450 (11 kDa), 377–450 (8 kDa), and 401–450 (6 kDa). The peptides containing aa 10–358 (pI 6.9) and 401–450 (pI 10.5) migrate as expected, whereas aa 359–450 (pI 4.9) and aa 377–450 (pI 5.5) migrate slower by 4 kDa, which may be explained by their low pI caused by stretches rich in D/E. Indeed, full-length N also migrates slower by 4 kDa (not shown). In all experiments (n = 17), we observed that the extent of digestion of the carboxy-terminus of N was dependent on the exact duration of the digestion. When we digested for a short time, most of N was cleaved after R358. However, after longer digestion, sequences starting at S377 and S401 were found (Figs. 1b and c).

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**Fig. 1.** Trypsin digestion of the N–P complex. The N–P complex was digested at 1:500 w/w with trypsin for 150 min at room temperature. The digestion was quenched, and the sample was loaded and eluted on a Superdex-75 HR10/30 column. The chromatograph of the eluate (in 0.2-ml fractions) is shown in panel a (upper line is A280 and lower line is A260) and the 16.5% Tris–tricine SDS-PAGE profile of peak I in panels b and c and peak II in panel d. The gels in panels b and c are from two independent experiments with a less extensive (in panel b) and a more extensive (in panel c) digestion of N. The N-terminal sequencing results of the bands are indicated. The gels were colored with Coomassie Blue.
In all cases, the digestion products of N migrated as a single peak on the Superdex-75 column with a reproducible elution volume of 10.2–10.5 ml (Fig. 1a, peak I), corresponding to the elution volume of a globular protein of ~55 kDa based on the calibration of the column with proteins of known size. The size of the intact N protein being 50 kDa, this indicates that the trypsin either made single cuts in exposed loops or removed short loops without affecting the overall structure of the protein. It was surprising that no P protein peptide could be detected together with the N fractions since P is assumed to be an essential chaperone to keep N monomeric. However, when peak II was analyzed (Figs. 1a and d), large parts of the phosphoprotein could be found. The bands corresponded to sequences of P starting at aa 72/77 and 173/177 or aa 187. Based on their apparent sizes, the bands starting at residues 173/177/187 could extend up to the carboxy-terminus at position 297. All encompass the domain of P that binds to N in the N–RNA. The band starting with aa 72/77 could correspond to the rabies virus equivalent of the stable domain of 70 residues (8.3 kDa) identified in the vesicular stomatitis virus P protein (Ding et al., 2004). Most of P is, thus, removed by the trypsin digestion of the N–P complex apparently without a change in the monomeric, soluble state of N.

In order to obtain a more homogeneous digestion of the nucleoprotein, we digested the purified trypsinized complex (Fig. 1a, peak I) with a second protease. Among the proteases tested (subtilisin, elastase, thermolysin, bromelain), only subtilisin seemed to further trim exposed loops without affecting the overall structure of N. Fig. 2a shows that the doubly digested N–P complex still migrates as a single peak on the Superdex-75 column with an apparent molecular weight of about 48 kDa. The SDS-PAGE profile of the peak (Fig. 2b) shows three bands that were all analyzed by mass spectrometry. While the trypptic fragment aa 401–450 was not further digested by subtilisin, aa 10–358 was further cleaved into two fragments (aa 25–119 and aa 130–356), each of which was trimmed at the extremities: At the amino-terminus of N, subtilisin further removed 15 residues from V10 up to Q25; in addition, residues W120-T129 and R357-R400 were removed (Fig. 2c). This implies that these residues must be exposed at the surface of N in the N–P complex, and that their removal does not have an influence on the integrity and folding of the rest of N.

The fact that we could purify soluble N protein without detecting any remaining peptides of the P protein in the same fractions by SDS-PAGE was intriguing. Either P was totally absent and its initial binding to N in the N–P complex had...
conferring a stable conformation to the nucleoprotein keeping N soluble, or the remaining part of P is a very small peptide that was not detectable by SDS-PAGE and Coomassie staining. We thus tried immunoblotting of a gel like the one shown in Fig. 2b (doubly digested N–P complex), using the following monoclonal antibodies directed against different regions of P: 25E6 (ag. aa 1–6), 23E2 (ag. aa 20–52), 30F2 (ag. aa 83–138), 25C2 (ag. aa 225–246), and 26G6 (ag. aa 267–297) (Raux et al., 1997). From all MAbs tested, only MAb 23E2 gave a weak positive result in two independent assays using a colorimetric alkaline phosphatase assay (data not shown). The detected P peptide migrated at about the same position as the N 401–450 peptide. This prompted us to sequence this band after transferring to a PVDF membrane, and apart from the sequence of N, we also found a sequence corresponding to residues 4–429 (IFVNPSAIRA-13 of P. We subsequently performed denaturing mass spectrometry on the doubly digested N–P complex. We found a peak corresponding to a quadruply charged P peptide which was subsequently sequenced by tandem mass spectrometry and identified as residues 4–40 (IFVNPSAIRA-GLADLEMAEETVLDLRRNIEDNQAHLLQ) of P. Peptides corresponding to residues 14–40 and 22–30 were also detected, as triply and doubly charged peptides, respectively. Therefore, both the N-terminal sequencing results and the mass spectrometry data show that an amino-terminal peptide of the phosphoprotein, made up of residues 4–40 (4 kDa), is strongly associated with the nucleoprotein in the N–P complex even after double digestion with trypsin and subtilisin and purification over size-exclusion columns after each digestion.

We next investigated whether the amino-terminal P peptide alone was able to bind to N in vivo. For these experiments, we chose to use a peptide corresponding to the first 42 residues of P and one that contained the first 60 residues because the sequence conservation between lyssavirus phosphoproteins extends to about residue 60 (Fig. 5). BSR cells, infected with vTF7.3 recombinant vaccinia virus, were transfected with plasmids coding for N alone (no tag), for N plus full-length P virus, the 42 amino-terminal residues of P plus a FLAG tag, or N plus P60 (the 60 amino-terminal residues of P plus a FLAG tag), all under the control of a T7 promoter. The cell lysates were analyzed 24 h post-transfection by SDS-PAGE and Western blotting using a rabbit polyclonal antibody against rabies virus nucleocapsid that recognizes both N and P (Fig. 3 lanes 1–4). The amounts of N in the lysates were globally equivalent, but less N was observed upon coexpression of P60 (lane 4). Full-length P was also detected (lane 2), while the P-42 and P-60 peptides were too small to be retained on the Tris–glycine gel used (lanes 3 and 4). Next, the cell lysates were immunoprecipitated with an anti-FLAG antibody, and the precipitates were analyzed by Western blot (Fig. 4, lanes 5–8), N was coprecipitated both with P42 and P60, indicating that the first 42 and 60 amino-terminal amino acids of P alone are able to bind to N.

Finally we performed a yeast two-hybrid experiment in which we tested various combinations of the Gal4-DNA-binding domain (BD) fused to N, P, P42, and P60 against the Gal4-activating domain (AD) fused to P, P42, and P60 (Fig. 4). The interaction between N and P was as strong as that of N with P60, whereas the interaction between N and P42 was clearly established, but somewhat less strongly. While P confirmed its strong potential to self-oligomerize (Gigant et al., 2000; Jacob et al., 2001), neither P42 nor P60 interacted with P indicating that the oligomerization domain is located at a more central location on P.

**Discussion**

Fig. 5 shows a multiple sequence alignment for the first 116 aa residues of the phosphoproteins of lyssaviruses (rhabdovirus genus that contains rabies virus), as well as a secondary structure prediction. Apart from strong sequence conservation within the first 60 residues and within the carboxy-terminal N–RNA-binding domain of P, most of the sequence of the lyssavirus phosphoproteins between residues
60 and 200 is highly variable (Fig. 1 in Mavrakis et al., 2004). Residues 4–40 of the phosphoprotein are predicted to form an α-helix and contain seven conserved aspartates/glutamates (four of which are strictly conserved). There are strictly conserved proline (Pro8) and glycine (Gly41) residues at the ends of this peptide (with a strictly conserved glycine at position 14) and also a strictly conserved arginine at position 12. The fact that the peptide was not cleaved with trypsin at this site indicates that R12 is hidden in the N–P complex.

The identified P peptide (4–40 aa) with the conserved acidic residues is negatively charged with a p\(\text{I}\) of 4.2. We speculate that the negative charge of the peptide mimics the sugar-phosphate backbone of RNA, and that the peptide binds to the RNA-binding site of N, thus preventing N from binding to cellular RNA.

The 52 amino-terminal residues of the phosphoprotein are also engaged in binding to the viral polymerase (Chenkik et al., 1998; Jacob et al., 2001), of which the first 19 aa are most critical. This means that binding of P to N in the N–P complex would interfere with P binding to the polymerase. It is also possible that binding of P to the polymerase competes with binding of P to N in the N–P complex. In this way, the replicating polymerase could displace P and use the newly available N for encapsidation of newly made viral RNA.

In the yeast two-hybrid experiment, P does not form an oligomeric complex with P42 or P60. Previous work has suggested that the oligomerization domain for rabies virus P is situated between residues 93 and 130, a region that is also predicted to be helical (Mavrakis et al., 2004). This domain in rabies virus P could be related to the domain between residues 107 and 178 of VSV P that was identified by limited protease digestion and appears to be oligomeric (Ding et al., 2004). Because P42 and P60 bind to N in the N–P complex independently of the rest of P, they probably bind as monomeric peptides. The reason why the N–P complex contains one molecule of N and two molecules of P (Mavrakis et al., 2003) is not clear, but the most likely explanation is that the phosphoprotein itself is a dimer (Gao and Lenard, 1995). It is possible that only a single amino-terminal peptide of the dimer is bound to N, or that two peptides are bound at different sites on N. The peptide that was isolated from the doubly digested N–P complex ended at residue 40. From the two-hybrid experiment, however, it is clear that the P42 peptide does not bind as strongly to N as intact P or P60 that give about the same signal (Fig. 4). This suggests that, if in the intact N–P complex residues 1–60 of P do bind to N, the last 20 residues are less protected against proteolytic digestion than the first 40 residues. A 60 aa peptide is probably folded in a more complex manner than a single α-helix. This could mean that the first 40 residues are part of a larger domain that binds to N, but that residues 4–40 are closest to N and thus most protected against proteolytic digestion. Residues 41–60 could stabilize the folding of the domain and its interaction with N.

The N–RNA-binding domain of P is removed from the N–P complex after the first trypsin digestion step, indicating that this domain of P is not bound to N in the N–P complex. Thus, the amino-terminal domain of P is used only for binding to N in the N–P complex, whereas the carboxy-terminal domain of P is used only for binding to N in N–RNA when P functions as a cofactor of the viral polymerase (Mavrakis et al., 2004).

The peptide of Sendai virus P that binds to N in the N–P complex seems to be much shorter than the rabies virus peptide. There is no obvious sequence similarity between the peptides although both contain a number of conserved acidic amino acids (Curran et al., 1995). It is remarkable that both viruses seem to use P peptides in a similar manner to prevent N from non-specific binding to cellular RNA, in particular because the carboxy-terminal parts of the respective phosphoproteins are not homologous. At this moment, it is not known if other NNSR viruses employ the same mechanism to keep their nucleoproteins from binding to cellular RNA.
Materials and methods

Expression and purification of the N–P complex

The N–P complex (rabies virus strain CVS) was produced and purified as described in Mavrakis et al. (2003) with the only difference that High Five insect cells were used in this study because the yield was higher than when Sf21 cells were used.

Preparative digestion and subsequent purification of the N–P complex

The N–P complex was digested with trypsin at a trypsin:trypsin ratio of 1:500 w/w for 150 min at 22–23 °C after which the digestion was stopped by the addition of PefablocSC plus PSC-Protector Solution (Roche Diagnostics). The sample was then concentrated and loaded on a Superdex 75 HR10/30 column and eluted in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM DTT (buffer A). Fractions were analyzed by 16.5% Tris–tricine SDS-PAGE. The gel was incubated for 30 min in a glutaraldehyde fixing solution (0.2% v/v glutaraldehyde, 30% v/v ethanol, 0.2 M sodium acetate) for better visualization of proteins with a M<sub>c</sub> < 10 kDa. N-containing fractions were pooled, and the protein concentration was determined by the Bradford assay. The sample was then digested with subtilisin at a subtilisin:protein ratio of 1:50 w/w for 190 min at 22 °C after which the digestion was quenched as for trypsin. The sample was concentrated and loaded on a Superdex 75 HR10/30 column and eluted in buffer A. Fractions were analyzed by 16.5% Tris–tricine SDS-PAGE, the gel cross-linked and stained, and the N-containing fractions pooled and used for further analysis.

Mass spectrometry

Mass spectrometry was performed by Xinping Li and Thomas Franz, EMBL, Heidelberg, Germany. The solution was firstly desalted with ZipTip C18 (Millipore): the sample was adsorbed to the wetted (50% acetonitrile) and equilibrated with 0.1% trifluoroacetic acid) ZipTip, washed with 0.1% trifluoroacetic acid and finally eluted with 50% acetonitrile/45% water/5% formic acid. The desalted sample was then filled in a nano-electrospray needle and applied to a Mass Spectrometer Q-TOF 2 (Waters, previously Micromass, Manchester, UK). The spectra were acquired under the following conditions: ionization mode: ES<sup>+</sup>; capillary voltage: 1.5 kV; source temperature: 100 °C; cone energy: 40 eV; collision energy: for MS 8 eV, for MS/MS 30–55 eV depending on individual precursors; mass range: for MS 500–2000 m/z, for MS/MS 50–2500 m/z, depending on individual precursors.

N-terminal sequence determination

N-terminal sequencing was done by Jean-Pierre Andrieu, IBS, Grenoble, France. Amino acid sequence determination based on Edman degradation was performed using an Applied Biosystems gas-phase sequencer model 492 (s/n: 9510287J).

Phenylthiohydantoin amino acid derivatives generated at each sequencing cycle were identified and quantified on-line with an Applied Biosystems Model 140C HPLC system using the data analysis system for protein sequencing from Applied Biosystems Model 610A (software version 2.1). The PTH-amino acid standard kit (Perkin Elmer P/N 0101016) was used and reconstituted according to the manufacturer’s instructions (900776 Rev D). The procedures and reagents used were as recommended by the manufacturer. Retention times and integration values of peaks were compared to the chromatographic profile obtained for a standard mixture of derivatized amino acids.

Plasmid constructions

Fusion proteins used as bait and prey in the two-hybrid assays contained the relevant ORF fused C-terminal of (1) the Gal4p transcription activation domain (AD) cloned in pACTII (Clontech); (2) the Gal4p-DNA-binding domain (BD) cloned in pAS2ΔΔ (Jacob et al., 2001) for the full-length N or P proteins or in pGBK7 (Clontech) for the first 42 (P-42) or 60 (P-60) NH<sub>2</sub> amino acids of the P protein. N and P proteins were from the rabies PV strain (Tordo et al., 1986). Full-length N and P constructs were previously described (Jacob et al., 2001). The coding regions for P-42 and P-60 were cloned with Gateway technology (Invitrogen) using primers:

P-42 f: 5′-GGGGACAGTGGGTCACAAAAGACGGG-GTTCACCATGAGCAAGATTTGTCAATCC-3′
P-42 r: 5′-GGGGACCATTGTTACAAAGAGCTGAGTCTCCTAGGGTTCCCTTGAGAGTACCC-3′
P-60 f: 5′-GGGGACAGTGGGTCACAAAAGACGGG-GCTTCACCATGAGCAAGATTTGTCAATCC-3′
P-60 r: 5′-GGGGACAGTGGGTCACAAAAGACGGG-GCTTCACCATGAGCAAGATTTGTCAATCC-3′

in which Gateway recombinant regions are underlined and P-specific coding regions are in italics. For T7-driven peptide expression, peptides P-42 and P-60 were cloned into plasmid pCINeo3Flag (Sigma modified with the Gateway cassette), which added 3 Flag sequences (Sigma, 3 × DYKDDDDK) to the amino-terminus of the peptide (Jacob et al., 2001). Plasmids pT7-N and pT7-P encoding the full-length N and P protein in a pBluescript II KS (+) (pBl, Stratagene) vector were described earlier (Le Mercier et al., 2002). Cloning junctions and ORFs were verified by sequencing on an ABI 377 automatic analysis system for protein sequencing from Applied Biosystems Model 610A (software version 2.1). The PTH-amino acid standard kit (Perkin Elmer P/N 0101016) was used and reconstituted according to the manufacturer’s instructions (900776 Rev D). The procedures and reagents used were as recommended by the manufacturer. Retention times and integration values of peaks were compared to the chromatographic profile obtained for a standard mixture of derivatized amino acids.

Yeast two-hybrid analysis

Various combinations of plasmids encoding GAL4BD-X and GAL4AD-Y were transformed into S. cerevisiae Y187/CG1945 strain using the LiCl procedure as described earlier (Jacob et al., 2001). After streaking the resulting colonies on plates of SD minimal medium, a β-galactosidase activity assay
was performed by covering the plates with a gel containing 0.25 M Na₂HPO₄ pH 7.5, 0.5% agar, 0.1% SDS, 7% dimethylformamide, 0.04% X-Gal (Euromedex). The intensity of the blue color on each colony is indicative of the strength of the interaction between the two partners.

Expression of recombinant N, P, and P mutants in BSR cells

BSR cells were plated (2.5 × 10⁵ cells per well) in a 6-well plate in Dulbecco’s modified Eagle’s Medium, Glutamax I (DMEM, Gibco) supplemented with 8% fetal calf serum (FCS). After 24 h at 37 °C (5% CO₂), the medium was removed, and the cells were infected for 30 min at 37 °C with T7 recombinant vaccinia virus vTF7-3 (MOI 10 pfu/cell) in DMEM to allow cytoplasmic expression of T7-RNA polymerase. The medium was then removed, and the cells were transfected with various combinations of the plasmids pT7-N, pT7-P, pClNeo3Flag-P42, pClNeo3Flag-P60 (3 μg each/well) using polyethyleneimine (PEI, Aldrich) as described earlier (Jacob et al., 2001). After 24 h at 37 °C (DMEM, 5% FCS, 5% CO₂), the medium was removed, and each well was treated with 1 ml of lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.5% Igepal (Sigma)). Lysates were clarified by centrifugation at 3000 rpm for 3 min at 4 °C.

N–P coimmunoprecipitation, SDS-PAGE analysis, and immunoblotting

Fifteen microliters of agarose beads with G and A proteins was washed 3 times in lysis buffer and then incubated with 1 μg of anti-Flag antibody for 8 h at 4 °C under agitation. After 3 washings in lysis buffer, beads were incubated overnight at 4 °C under agitation with 50 μl of BSR cell lysates diluted in 200 μl of 50 mM Tris–HCl pH 8, 150 mM NaCl. After 4 washings in lysis buffer, 20 μl of Laemmli buffer (80 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and bromophenol blue) was added to the beads.

Total and FLAG-immunoprecipitated cell lysates were separated by SDS-PAGE and then electrotransferred on nitrocellulose (Hybond ECL, Amersham Bioscience). The membrane was saturated overnight at 4 °C in 3% BSA-PBS, washed 3 times in 0.2% Tween20-PBS, and then subjected to sequential incubations first with a rabbit anti-RNP polyclonal antibody (dilution 1/5000) in 3% BSA-PBS for 1 h at room temperature, followed by three washes in 0.2% Tween20–PBS, and then with a goat anti-rabbit antibody conjugated to horseshadish peroxidase (Vector, dilution 1/10,000) in 3% BSA-PBS for 1 h at room temperature. The membrane was finally washed 3 times in 0.2%. Tween20–PBS, and revelation was performed on Hyperfilm MP (Amersham Bioscience) using the ECL Super signal kit (Pierce).

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