Pokeweed Antiviral Protein Accesses Ribosomes by Binding to L3

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Pokeweed antiviral protein (PAP), a 29-kDa ribosome-inactivating protein, catalytically removes an adenine residue from the conserved a-sarcin loop of the large rRNA, thereby preventing the binding of eEF-2-GTP complex during protein elongation. Because the a-sarcin loop has been placed near the peptidyltransferase center in Escherichia coli ribosomes, we investigated the effects of alterations at the peptidyltransferase center on the activity of PAP. We demonstrate here that a chromosomal mutant of yeast, harboring the mak8–1 allele of peptidyltransferase-linked ribosomal protein L3 (RPL3), is resistant to the cytostatic effects of PAP. Unlike wild-type yeast, ribosomes from mak8–1 cells are not depurinated when PAP expression is induced in vivo, indicating that wild-type L3 is required for ribosome depurination. Co-immunoprecipitation studies show that PAP binds directly to L3 or Mak8–1p in vitro but does not physically interact with ribosome-associated Mak8–1p. L3 is required for PAP to bind to ribosomes and depurinate the 25 S rRNA, suggesting that it is located in close proximity to the a-sarcin loop. These results demonstrate for the first time that a ribosomal protein provides a receptor site for an ribosome-inactivating protein and allows depurination of the target adenine.

The 29-kDa pokeweed antiviral protein (PAP) isolated from the American pokeweed plant, Phytolacca americana is a single-chain ribosome-inactivating protein (RIP) that catalytically removes a specific adenine residue from the highly conserved, surface-exposed, a-sarcin loop in the large rRNA of eukaryotic and prokaryotic ribosomes (1, 2). PAP displays broad-spectrum antiviral activity against plant and animal viruses, including influenza virus (3), poliovirus (4), herpes simplex virus (5), and human immunodeficiency virus (6). PAP removes an adenine base by cleavage of the N-glycosidic bond at A4324 in rat 28 S rRNA and at homologous sites on ribosomes from other organisms. Ribosomes depurinated in this manner are unable to bind the eEF-2-GTP complex, and protein synthesis is blocked at the translational step (7, 8). We previously reported that in vivo induction of PAP expression in yeast had a cytostatic effect. Growth was strongly inhibited when PAP expression was induced, possibly because of inhibition of translation (9). Because ribosomal frameshifting occurs during the elongation phase of protein synthesis, we investigated the role of translational inhibition by PAP in programmed ribosomal frameshifting by utilizing two different viral systems (the L-A and M1 “killer” system and the Ty1 retrotransposable element) of Saccharomyces cerevisiae. We demonstrated that expression of PAP in yeast leads to specific inhibition of programmed ribosomal frameshifting in the +1 direction and interferes with the ability of Ty1 to retrotranspose (10).

The rRNA substrate for PAP, the a-sarcin loop, has been localized in close proximity to the peptidyltransferase center within the 50 S subunit of Escherichia coli ribosomes (11). We hypothesized that yeast cells harboring mutations in ribosomal proteins that have been linked to the peptidyl transfer reaction may be resistant to the cytostatic effects of PAP. One of these mutants, mak8–1, was discovered by its inability to maintain the M1 satellite virus (12), mak8–1 is an allele of RPL3/TCM1 (13), which encodes the large ribosomal subunit protein L3 (14). L3 has been shown to participate in the formation of the peptidyltransferase center (15, 16) and identified as an essential protein in the catalysis of peptide bond formation (17). Recent results indicate that strains harboring the mak8–1 allele of RPL3 exhibit increased programmed frameshifting efficiencies, supporting the notion that events at the peptidyltransferase center play a critical role in programmed ribosomal frameshifting (18). The current study demonstrates for the first time that ribosomal protein L3 is essential for binding of PAP to ribosomes and subsequent depurination of the a-sarcin loop, providing direct evidence that PAP accesses its rRNA substrate by binding to a ribosomal protein.

EXPERIMENTAL PROCEDURES

Yeast Strains and Vectors—The cDNAs encoding PAP (NT188) and PAPx (NT224) were introduced as SmaI/BglII fragments into the yeast expression vector YEp351. PAPx is an active-site mutant of PAP with a point mutation (E176V) that abolishes enzymatic activity (9). Transcription of the cDNAs was under the control of a galactose-inducible GAL1 promoter. Vectors containing PAP or PAPx were transformed (19) into the yeast strains S. cerevisiae W303 (MATa, ade2-1 trpl-1 ura3–1 leu2–3, 112 his3–11, 15 can1–100), 1906 (MATa, leu2 mak8–1), or the isogenic strains JD980 (MATa his2–2 his3–1 ura3–1 leu2–3, trp1Δ RPL3Δ his3G), containing either pRPL3 or pmak8–1 (18). YEp351 transformed into all cell types was used as a negative control.

Yeast Growth and Time Course Induction—Yeast cells were grown in 300 ml of H-Leu medium (20) with 2% raffinose at 30 °C to an absorbance of 0.6. Aliquots for protein analysis (2 ml), RNA extraction (15 ml), and ribosome isolation (25 ml) were removed and pelleted by centrifugation at 2,000 x g for 5 min. The remaining culture was pelleted at the same speed, washed in H-Leu medium, and resuspended in H-Leu medium with 2% galactose to induce the expression of PAP and PAPx. At various times during induction (2, 4, 8, 12, and 24 h), aliquots were removed, pelleted, and stored at –80 °C. Pellets for ribosome isolation were...
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RESULTS

mak8–1 Cells Are Resistant to PAP—PAP removes a specific adenine residue from the e-sarcin loop of yeast 25 S rRNA. Because this loop is located near the peptidyltransferase center, we introduced PAP into the strain harboring the mak8–1 allele to determine whether this mutation conferred resistance to the cytostatic effects of PAP. Both wild-type and mak8–1 cells were transformed with the LEU2-based vector, pNT188, containing the PAP cDNA under the control of a GAL1 promoter. As shown in Fig. 1, galactose induction of PAP expression did not have a cytostatic effect on the growth of the strain harboring the mak8–1 allele. In contrast, growth of wild-type cells was significantly inhibited when PAP expression was induced by galactose. To confirm this observation, isogenic RPL3::hisG strains (18) were tested for their sensitivity to PAP. Cells harboring pRPL3 were able to grow under PAP induction, whereas cells containing pRPL3 encoding wild-type L3 did not grow (data not shown).

PAP Is Expressed in mak8–1 Cells—Resistance to PAP may have arisen because either transcript or protein had not accumulated in mak8–1 cells. To determine whether PAP transcripts were synthesized, nucleic acid protection assays were performed to examine the accumulation of PAP mRNA relative to CYH2 mRNA, an internal control that encodes the ribosomal protein L29 (25). The levels of PAP transcript were compared with those of PAPx, the active-site mutant of PAP. The zero hour time point represents cells grown in raffinose under non-inducing conditions. As shown in Fig. 2, cells grown in raffinose did not express PAP or PAPx transcripts. However, 2 h after shifting to galactose-containing medium, transcripts corresponding to both PAP and PAPx were detected in mak8–1 cells. Quantitation of PAP mRNA, relative to the CYH2 internal control, indicated that both PAP and PAPx transcript levels remained constant during the time course of induction. A protected RNA fragment corresponding to PAP was not observed in cells containing the vector alone, 8 h after induction by galactose (Fig. 2, lane VC). When rRNA was used in place of total cellular RNA as a control, no specific binding by the radiolabeled probes was detected (Fig. 2, lane tRNA). These results demonstrated that both PAP and PAPx mRNAs were transcribed in cells harboring the mak8–1 allele, and no sig-
significant difference in the level of transcripts could be detected in cells expressing PAP or PAPx.

To test whether PAP was expressed and accumulated in mak8–1 cells, immunoblot analysis was conducted on aliquots harvested from cells grown on galactose medium through a 24-h time course. The same experiment was carried out with wild-type yeast cells harboring PAP (NT188) and PAPx (NT224). Similar amounts of PAP and PAPx were expressed in mak8–1 cells, suggesting a lack of toxicity due to PAP accumulation, whereas in wild-type cells, PAPx was expressed to a greater degree than PAP (Fig. 3). The higher molecular mass protein reacting with the PAP antibody likely represents the precursor form of PAP, observed previously in yeast (9). Overexpression of PAPx often results in lower molecular mass proteins, most likely breakdown products, seen clearly in wild-type cells induced for 24 h. However, the primary band in each immunoblot is the 29-kDa mature form of PAP.

mak8–1 Ribosomes Are Not Depurinated by PAP—To determine whether there were differences between the ability of PAP to depurinate ribosomes from wild-type and mak8–1 cells in vivo, ribosomes were isolated from yeast cells induced to express PAP or PAPx for 8 h. rRNA was isolated from these ribosomes, treated with aniline, and separated on a urea-acrylamide gel. Depurination of rRNA was revealed by the presence of the 360-nucleotide fragment produced by removal of a purine residue from the 25 S rRNA and subsequent cleavage at that site by treatment with aniline (arrow in Fig. 4). A positive standard for depurination was generated by incubating wild-type ribosomes with PAP in vitro, extracting the rRNA, and treating it with aniline (Fig. 4, lane std). Ribosomes isolated from wild-type cells harvested 8 h after induction of PAP expression were depurinated, whereas ribosomes of cells harboring the mak8–1 allele were not depurinated during PAP expression in vivo (Fig. 4.). Ribosomes isolated from both cell types expressing PAPx were not depurinated, which was consistent with the prior observation that PAPx lacks enzymatic activity.

PAP Does Not Associate with Ribosomes in mak8–1 Cells—A possible reason for the lack of depurination of rRNA in mak8–1 cells was that PAP may not be able to access its rRNA substrate in these cells. To determine whether PAP associated with ribosomes in wild-type cells, ribosomes examined for depurination were also assessed by immunoblot analysis with the affinity-purified antibody against PAP. As illustrated in Fig. 5A, both PAP and PAPx were associated with ribosomes in wild-type cells. In contrast, neither PAP nor PAPx could be detected with ribosomes isolated from mak8–1 cells. The higher levels of PAPx associated with ribosomes of wild-type cells likely reflected the increased level of expression of enzymatically inactive PAPx relative to the enzymatically active PAP (Fig. 3). The immunoblots of ribosomal proteins were stripped and reprobed with a monoclonal antibody against L3 to illustrate that L3 or its mutant form was detected on both types of ribosomes and that similar amounts of protein were loaded from both cell types (Fig. 5B).

PAP Binds Free L3 and Mak8–1p—Results described above indicated that PAP is associated with ribosomes in wild-type cells but not in mak8–1 cells, suggesting that PAP may interact with L3. To test the hypothesis of direct interaction with L3, purified PAP was mixed with in vitro synthesized L3 or Mak8–1p and co-immunoprecipitated with the monoclonal L3 antibody. Purified PAP co-immunoprecipitated with L3 or Mak8–1p when it was mixed with either protein and not when it was incubated alone (Fig. 6A). As expected, L3 and Mak8–1p were immunoprecipitated with L3 antibody when they were each mixed with PAP or incubated alone (Fig. 6B). These results indicated that PAP binds directly to L3 or Mak8–1p in its free form (Fig. 6A).
Ethidium bromide. A positive control was made by incubating 50 μg with ribosomes from wild-type (wt), (ribosomes was divided in half, and one-half was treated with aniline (mak8–1), and separated on a 7 M urea, 6% acrylamide gel and visualized with ribosomes after an 8-h induction. Total RNA extracted from 50 μg of ribosomes was divided in half, and one-half was treated with aniline (wt), the other half was not (mak8–1). After aniline treatment, RNA was separated on a 7 M urea, 6% acrylamide gel and visualized with ethidium bromide. A positive control was made by incubating 50 μg of wild-type ribosomes with 100 ng of PAP in vitro and treating with aniline (std), resulting in the 360-nucleotide rRNA fragment, shown by the arrow.

![Fig. 4. In vivo depurination of ribosomes from wild-type (wt) and mak8–1 cells. Expression of PAP or its active-site mutant (PAPx) was induced by growing the cells on H-Leu medium containing galactose. Wild-type cells containing vector alone (VC) were used as a negative control. Aliquots of cells (25 ml) were harvested for isolation of ribosomes. Total RNA extracted from 50 μg of ribosomes was divided in half, and one-half was treated with aniline (wt), the other half was not (mak8–1). After aniline treatment, RNA was separated on a 7 M urea, 6% acrylamide gel and visualized with ethidium bromide. A positive control was made by incubating 50 μg of wild-type ribosomes with 100 ng of PAP in vitro and treating with aniline (std), resulting in the 360-nucleotide rRNA fragment, shown by the arrow.](image1)

![Fig. 5. Immunoblot analysis indicating association of PAP with ribosomes from wild-type (wt) but not from mak8–1 cells. Ribosomes were isolated from both wild-type and mak8–1 cells after induction of PAP or its active-site mutant (PAPx) for 8 and 24 h. Ribosomes isolated from cells containing vector alone (VC) were used as a negative control. Total ribosomal protein (50 μg) was separated through 12% SDS-PAGE, transferred to nitrocellulose, and probed with an affinity-purified monoclonal antibody to PAP (1:5000). Purified PAP, 20 ng (Calbiochem), was used as a standard. B, the same blot as in A stripped with 8 M guanidine hydrochloride and reprobed with a monoclonal antibody to L3 (1:5000).](image2)

Co-immunoprecipitation of PAP and L3 from Ribosomes—To determine whether PAP interacts with L3 and Mak8–1p incorporated into ribosomes, ribosomes from wild-type or mak8–1 cells expressing either PAP, PAPx, or vector alone were immunoprecipitated with the monoclonal L3 antibody. As shown in Fig. 7A, PAPx was co-immunoprecipitated with L3 from ribosomes of wild-type, but not mak8–1 cells, indicating that PAPx does not interact with the mutant form of L3 in ribosomes. The lack of co-immunoprecipitation of PAP with L3 from ribosomes of wild-type cells may reflect the previous observation that wild-type protein is not synthesized as abundantly as the active-site mutant (Fig. 3). The difference may also be the result of variation in the kinetics of association between PAP and PAPx, namely PAP may dissociate more readily from its substrates, the ribosomes, than PAPx. Fig. 7B illustrates that L3 or Mak8–1p was immunoprecipitated from ribosomes of both cell types and that similar amounts of protein were loaded on the gel. These results suggest that the absence of association between PAP and Mak8–1p in ribosomes may be the result of a conformational change such that the peptide sequence or tertiary structure required is not accessible when Mak8–1p is incorporated into ribosomes. The lack of co-immunoprecipitation of PAP with Mak8–1p in ribosomes substantiates earlier results that showed the absence of PAP in ribosomes from mak8–1 cells and lack of rRNA depurination.

**FIG. 7. Co-immunoprecipitation of PAP with L3 from ribosomes.** Ribosomes (100 μg) isolated from wild-type (wt) and mak8–1 cells induced to express PAP, PAPx, or vector control (VC) for 8 h were incorporated into ribosomes. The lack of co-immunoprecipitation of purified PAP (Calbiochem) and 35S-labeled L3 and Mak8–1p, respectively, immunoprecipitated alone with the L3 antibody. Protein A-Sepharose beads were used as a background control (Bead).
cells are not associated with PAP, and consequently, are not depurinated.

The observation that PAP expressed in wild-type yeast depurinates ribosomes but does not when expressed in mak8–1 cells indicates that wild-type L3 is required for depurination of ribosomes. Co-immunoprecipitation experiments with isolated proteins illustrated that PAP directly binds to L3 and Mak8–1p in vitro. However, when the experiments were repeated using intact ribosomes, PAP co-immunoprecipitated only with wild-type L3 from ribosomes and not with Mak8–1p, indicating that PAP does not interact with Mak8–1p in ribosomes. The quaternary structure of a ribosome containing Mak8–1p may differ from a wild-type ribosome such that the binding site for PAP may be masked in the mutant ribosomes. Alternatively, a difference in post-translational modifications between L3 and Mak8–1p may affect its interaction with PAP in vivo. The hypothesis for altered binding by the mutant L3 is supported by the observation that we did not detect PAP or PAPx associated with ribosomes in mak8–1 cells; however, both proteins were associated with ribosomes in wild-type cells.

The evidence presented here demonstrates a link between L3 and the α-sarcin loop in eukaryotic ribosomes. Experiments designed to reconstitute the minimal ribosomal particle still capable of enzyme activity have established that L3 is essential for maintaining peptidyltransferase activity (15). A photolabile cDNA probe targeted to the central loop of domain V was shown to cross-link to L3 (26). With the use of a photolabile oligodeoxynucleotide probe complimentary to the α-sarcin region of E. coli, Muralikrishna et al. (11) recently demonstrated the proximity of the α-sarcin region to domains IV and V of E. coli tRNA. tRNA localization experiments further demonstrated the mutual proximity of domains IV, V, and VI within the 50 S subunit (27). Chemical and enzymatic footprinting have shown that L3 binds in region VIA of 23 S rRNA near the α-sarcin loop (28). Preliminary results from our laboratory indicate that both PAP and PAPx bind to the α-sarcin loop.2 The data presented here substantiate these observations by suggesting that L3 is in close proximity of the α-sarcin loop in yeast 25 S rRNA.

These data lead us to propose a model to explain the interaction between PAP and L3. Co-immunoprecipitation studies demonstrate that PAP binding to ribosomes requires wild-type L3. Therefore, we suggest that PAP accesses its substrate, the α-sarcin loop, by recognizing and binding to L3. Once bound, the close proximity of L3 to the α-sarcin loop would facilitate the subsequent depurination of the 25 S rRNA by PAP. Because PAPx does not interact with ribosomes from mak8–1 cells, we contend that the PAP binding site may be masked in mak8–1 ribosomes. The mak8–1 gene product encodes a mutant L3 that differs from the wild-type by only two amino acid substitutions, W255C and P257S, which may be sufficient to alter the shape of the protein product (18), affecting its interaction with other components of the ribosome. If rRNA is necessary to place the ribosomal proteins in a proper conformation to facilitate PAP binding, the point mutations in Mak8–1p may alter the interaction between rRNA and Mak8–1p.

Although the mechanism underlying the catalytic activity of RIPs is understood, very little is known about how RIPs gain access to the ribosome. Although all RIPs have the same specificity for adenine 4324 of naked 28 S rRNA, they show very different levels of activity against ribosomes of different species. For example, ricin is 23,000 times more active on rat liver ribosomes than on plant ribosomes (29), whereas PAP is equally active on ribosomes from all five kingdoms. These data suggest that the differences in sensitivity of ribosomes to RIPs may reflect differences in interactions of RIPs with ribosomal proteins. Endo and Tsurugi (30) showed that the ricin A chain depurinated rat rRNA at adenine 4324 in intact ribosomes much more efficiently than naked 28 S rRNA. Conversely, the ricin A chain depurinated naked 23 S rRNA of E. coli at the homologous adenine 2660 and did not depurinate intact E. coli ribosomes. Formation of a covalent complex between saporin and a component of the 60 S subunit of yeast ribosomes was shown by chemical cross-linking (31). Similarly, the ricin A chain has been cross-linked to mammalian ribosomal proteins L9 and L10e (32). Despite some evidence for the dependence of RIP activity on the type of ribosomal substrate, the functional significance of the association between RIPs and ribosomal proteins has not been reported. Nevertheless, these observations support the hypothesis for a molecular recognition mechanism involving one or more ribosomal proteins that could provide receptor sites for toxins and favor optimal binding to the target adenine. The results reported here demonstrate that PAP gains access to the ribosome by recognizing L3. Because L3 is highly conserved among ribosomes from different species, the interaction between PAP and L3 may be the underlying reason for the broad spectrum activity of PAP on ribosomes from different organisms.

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REFERENCES

1. Endo, Y., Tsurugi, K., and Lambert, J. M. (1988) Biochem. Biophys. Res. Commun. 150, 1032–1036
2. Hartley, M. R., Legname, G., Osborn, R., Chen, Z., and Lord, J. M. (1991) FEBS Lett. 290, 65–68
3. Tomlinson, J. A., Walker, V. M., Flewett, T. H., and Barclay, G. R. (1974) J. Gen. Virol. 22, 225–232
4. Usery, M. A., Irvin, J. D., and Hardesty, B. (1977) Ann. N. Y. Acad. Sci. 284, 451–460
5. Aron, G. M., and Irvin, J. D. (1980) Antimicrob. Agents Chemother. 17, 1032–1033
6. Zavitz, J. M., Moran, P. A., Haffar, O., Sia, J., Richman, D. D., Spina, C. A., Myers, D. E., Kuebelbeck, V., Ledbetter, J. A., and Uckun, F. M. (1991) Nature 347, 92–95
7. Montanaro, L., Sperri, S., Mattioli, S., Testoni, G., and Stirpe, F. (1975) Biochem. J. 146, 127–131
8. Osborn, R. W., and Hartley, M. R. (1990) Eur. J. Biochem. 193, 401–407
9. Hur, Y., Hwang, D.-J., Zoubenko, O., Coetzter, C., Uckun, F. M., and Tumer, N. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8448–8452
10. Tumer, N. E., Parikh, B. A., Li, P., and Dinman, J. D. (1998) J. Virol. 72, 1036–1042
11. Muralikrishna, P., Alexander, R. W., and Cooperman, B. S. (1997) Nucleic Acids Res. 25, 4562–4569
12. Wickner, R. B., and Liebowitz, M. J. (1974) Genetics 67, 432–432
13. Wickner, R. B., Porter-Ridley, S., Fried, H. M., and Ball, S. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4706–4708
14. Fried, H. M., and Warner, J. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 238–242
15. Hopf, H., Schulze, H., and Nierhaus, K. H. (1981) J. Biol. Chem. 256, 2284–2288
16. Noller, H. F. (1993) J. Bacteriol. 175, 5297–5300
17. Green, R., and Noller, H. F. (1997) Annu. Rev. Biochem. 66, 679–716
18. Peltz, S. W., Hammell, A. B., Cui, Y., Yasenchak, J., Poljanowski, L., and Dinman, J. D. (1999) Mol. Cell. Biol. 19, 384–391
19. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 463–468
20. Treco, D. A., and Lundblad, V. (1993) Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Greene and Wiley, Cambridge, MA
21. Cui, Y., Dinman, J. D., and Peltz, S. W. (1996) EMBO J. 15, 5726–5736
22. Lindstrom, J. T., and Belanger, F. C. (1994) Plant Physiol. 106, 7–16
23. Tumer, N. E., Hwang, D.-J., and Bonness, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3866–3871
24. Otto, J. J., and Lee, S.-W. (1983) Methods Cell Biol. 37, 119–126
25. Stocklein, W., and Piepersberg, W. (1980) Curr. Genet. 1, 177–183
26. Alexander, R. W., Muralikrishna, P., and Cooperman, B. S. (1994) Biochemistry 33, 8448–8452
27. Alexander, R. W., Muralikrishna, P., and Cooperman, B. S. (1994) Biochemistry 33, 8448–8452
28. Alexander, R. W., Muralikrishna, P., and Cooperman, B. S. (1994) Biochemistry 33, 8448–8452
29. Alexander, R. W., Muralikrishna, P., and Cooperman, B. S. (1994) Biochemistry 33, 8448–8452

P. Wang and N. E. Tumer, unpublished data.
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27. Joseph, S., and Noller, H. F. (1996) *EMBO J.* **15**, 910–916
28. Muralikrishna, P., and Cooperman, B. S. (1991) *Biochemistry* **30**, 5421–5428
29. Harley, S. M., and Beevers, H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 5935–5938
30. Endo, Y., and Tsurugi, K. (1988) *J. Biol. Chem.* **263**, 8735–8739
31. Ippoliti, R., Lendaro, E., Bellelli, A., and Brunori, M. (1992) *FEBS Lett.* **298**, 145–148
32. Vater, C. A., Bartle, L. M., Leszyk, J. D., Lambert, J. M., and Goldmacher, V. S. (1995) *J. Biol. Chem.* **270**, 12933–12940