silver particles were rendered red and superimposed on a bright-field image of the section. No substantial signal above background was apparent after hybridization with sense probes.
8. A. J. Fleming, T. Mandel, I. Roth, C. Kuhlemeier, *Plant Cell* **5**, 297 (1993); A. J. Fleming, T. Manzara, W. Gruissem, C. Kuhlemeier, *Plant J.* **10**, 745 (1996).
9. M. Snow and R. Snow, *Philos. Trans. R. Soc. London Ser. B* **221**, 1 (1931).
10. E. J. Szymkowiak and I. M. Sussex, *Plant Cell* **4**, 1089 (1992); S. Hantke, R. Carpenter, E. S. Coen, *Development* **121**, 27 (1995).
11. N. Wang, J. P. Butler, D. E. Ingber, *Science* **260**, 1124 (1993).
12. K. Roberts, *Curr. Opin. Cell Biol.* **6**, 688 (1994); C. Brownlee and F. Berger, *Trends Genet.* **11**, 344 (1995); R. S. Quatrano and S. L. Shaw, *Trends Plant Sci.* **2**, 15 (1997).
13. D. V. Basile, in *Bryophyte Development and Physiology*, R. N. Chopra and S. C. Bhatla, Eds. (CRC Press, Boca Raton, FL, 1990), pp. 225–243.
14. A. J. De Jong *et al.*, *Plant Cell* **4**, 425 (1992).
15. An expansin clone was isolated from meristem cDNA (*8*) by reverse transcription and the polymerase chain reaction with primers designed from the published sequences (*4*). This cDNA fragment (expansin 18), which showed 73.8% identity to the cucumber sequence CuExS1 over the amplified 144 base pairs, was used as a substrate for riboprobe synthesis and in situ hybridization (*7*).
16. P. Reymond, S. Grünberger, K. Paul, M. Müller, E. E. Farmer, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4195 (1995).
17. We thank A. Buchala and E. Farmer for endocellulase and oligogalacturonic acid, respectively; I. Sussex, P. Green, C. Smart, and I. Dupuis for constructive criticism; H. Imboden for use of the confocal imaging facility; and M. Kummer for scientific artwork. Supported by a Swiss National Science Foundation grant to C.K. and A.J.F.

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STAT3 as an Adapter to Couple Phosphatidylinositol 3-Kinase to the IFNAR1 Chain of the Type I Interferon Receptor

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STAT (signal transducers and activators of transcription) proteins undergo cytokine-dependent phosphorylation on serine and tyrosine. STAT3, a transcription factor for acute phase response genes, was found to act as an adapter molecule in signal transduction from the type I interferon receptor. STAT3 bound to a conserved sequence in the cytoplasmic tail of the IFNAR1 chain of the receptor and underwent interferon-dependent tyrosine phosphorylation. The p85 regulatory subunit of phosphatidylinositol 3-kinase, which activates a series of serine kinases, bound to phosphorylated STAT3 and subsequently underwent tyrosine phosphorylation. Thus, STAT3 acts as an adapter to couple another signaling pathway to the interferon receptor.

Interferons (IFNs) are cytokines that block the viral infection of cells, inhibit cell proliferation, and modulate cell differentiation. Type I IFNs (IFN-α, -β, and -ω) compete with each other for binding to a common cell surface receptor (IFN-R), distinct from the receptor for type II IFN (IFN-γ) (*1*). The IFN-R is composed of IFNAR1 and IFNAR2 chains (*2–4*). The IFNAR1 chain undergoes rapid ligand-dependent tyrosine phosphorylation and acts as a species-specific transducer for the actions of type I IFN, which suggests that it has a central role in signaling through the IFN-R (*5–7*). IFNs cause transduction of a signal to the nucleus that results in selective stimulation of the IFN-stimulated genes (ISGs) (*8–10*). Transcriptional activation of ISGs is mediated by the protein tyrosine kinase–dependent phosphorylation of the STAT latent cytoplasmic transcriptional activators (*11, 12*). Upon tyrosine phosphorylation, IFN-α–activated STATs (STAT1, STAT2, and STAT3) form homo- and heterodimers.

Although tyrosine phosphorylation of STATs and IFN-R are important early events in IFN signaling, serine phosphorylation events are also necessary for the induction of IFN action (*13–16*). Here, IFN-α induced the rapid tyrosine phosphorylation of the regulatory 85-kD (p85) subunit of phosphatidylinositol 3-kinase (PI 3-kinase), an upstream element in a serine kinase transduction cascade (*17, 18*). IFN-α–dependent recruitment of p85 to the IFNAR1 chain of the IFN-R required the tyrosine phosphorylation of the YSSQ and YSNE motifs that are present in the conserved IRTAM (IFN receptor tyrosine activation motif) cytosolic sequence KYSSQTSQDSGNYSNE in IFNAR1 (*19*). The IRTAM functions in the signaling through the IFN-R by specifically acting as a docking site for cytoplasmic proteins containing the Src homology 2 (SH2) domain (*6, 20*). Interaction of p85 with IFNAR1 required STAT3 phosphorylation at Tyr^{656} [a YXXM motif (*21*), a known consensus binding site for the SH2 domains of p85].

The IFNAR1 chain undergoes IFN-dependent tyrosine phosphorylation, and several tyrosine-phosphorylated proteins coprecipitate with the IFNAR1 chain (*6, 22*). To determine whether p85 interacts with IFNAR1, we precipitated proteins from lysates of control and IFN-treated Daudi cells with an IFNAR1-specific antibody and analyzed them by blotting with antiserum to p85 (Fig. 1A). Although similar amounts of IFNAR1 chain were immunoprecipitated from treated or untreated cells (*23*), only precipitates from IFN-α–treated cells contained p85. Maximal association was observed at 5 min after IFN addition and decreased rapidly thereafter. IFNAR1 and STAT3 also coprecipitated with p85 in lysates from IFN-α–treated cells (Fig. 1B).

These results led us to investigate whether the tyrosine-phosphorylated IFNAR1 chain bound p85. The phosphorylation of conserved motifs present in the IFNAR1 subunit may create sites for high-affinity interactions with cytoplasmic proteins containing SH2 and phosphotyrosine binding (PTB) domains (*17, 18, 24, 25*). STAT3 directly binds to the tyrosine-phosphorylated IFNAR1 chain through its SH2 domain (*22*); thus, the IFNAR1 chain might also interact with the SH2 domains of p85. We prepared glutathione-S-transferase (GST) fusion proteins that encompassed the NH_2-terminal (Np85), COOH-terminal (Cp85), or both SH2 domains of p85 (N+Cp85). Lysates from IFN-α–treated Daudi cells were incubated with GST fusion proteins bound to glutathione-agarose beads. The precipitated material was analyzed by blotting with anti-IFNAR1. The fusion protein containing both SH2 domains of p85 precipitated much greater amounts than did fusion proteins containing only one SH2 domain (Fig. 2). The N+Cp85 fusion protein did not precipitate IFNAR1 from lysates from control cells or cells pretreated with the tyrosine kinase inhibitor genistein, which demonstrated that tyrosine phosphorylation is required for interaction of IFNAR1 with p85 fusion proteins. IFNAR1 did not interact with GST protein alone (*22*). N+Cp85 also precipitated tyrosine-phosphorylated STAT3 from lysates prepared from IFN-α–treated cells.

These results led us to determine whether STAT3 or IFNAR1 could directly interact with p85, as measured by direct blotting with the N+Cp85 fusion protein. Although N+Cp85 did not directly bind to tyrosine-phosphorylated IFNAR1, it did bind to tyrosine-phosphorylated STAT3 (Fig. 2C). These results suggest that a strong interaction of p85 with IFNAR1 requires both SH2 domains of p85 and that such an interaction is indirect because p85 binds directly to STAT3 but not IFNAR1. However, additional adapt-

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ers (for example, GRB2 binding to the YXNX motif of the IRTAM) could also be involved in mediating the indirect interaction between IFNAR1 and p85.

STAT3 binds to the YXXQ motif present in the gp130 signal-transducing chain of the interleukin-6 receptor family (*26*). The YXXQ and YXXE motifs are present within the IRTAM of IFNAR1's cytoplasmic tail (*22*). To determine whether either of these intracellular tyrosine residues is required for the interaction of p85 with the IFNAR1 chain, we introduced peptides corresponding to the amino acids surrounding these two tyrosine residues into Daudi cells permeabilized with streptolysin O. The permeabilized cells were treated with IFN-α and then assayed for the interaction of the IFNAR1 chain with p85. Phosphopeptides corresponding to Tyr^{527} (YSSQ motif) or Tyr^{538} (YSNE motif) blocked the IFN-induced interaction of IFNAR1 with p85 (Fig. 3). In contrast, nonphosphorylated peptides, a phosphopeptide corresponding to the membrane proximal Tyr^{481}, and an irrelevant phosphopeptide had no effect on coprecipitation of p85. These results indicated that the interaction of p85 with IFNAR1 occurs through the conserved YSSQ and YSNE motifs in the IRTAM. A consensus YXXM p85-binding motif is present in STAT3 at Tyr^{656} (this motif is conserved in mouse and human STAT3 homologs), whereas no such motif is found in IFNAR1. This led us to investigate the possible role of STAT3 as an adapter that couples p85 to IFNAR1.

Wild-type STAT3 and an F656-STAT3 point mutant (in which a phenylalanine was substituted for Tyr^{656} of the YXXM motif) were cloned into the pcDEF1 expression vector (*27*) and were electroporated, along with the IFNAR1 chain, into COS-7 cells. We assessed STAT3's association with IFNAR1 and the activation of transfected STAT3 by precipitating proteins from IFN-treated cell lysates with anti-IFNAR1 or anti-STAT3 and blotting with anti-STAT3 or anti-pTyr, respectively. Cells expressing F656-STAT3 had very low amounts of IFN-induced STAT3 tyrosine phosphorylation relative to that of cells expressing wild-type STAT3 (Fig. 4A). We assayed the IFN-induced association of p85 with IFNAR1 in cells cotransfected with IFNAR1 and either wild-type STAT3 or F656-STAT3 constructs. Although IFN treatment of cells expressing wild-type STAT3 resulted in coprecipitation of p85 with IFNAR1, IFN-treated cells transfected with F656-STAT3 yielded no detectable p85 coprecipitated with IFNAR1 (Fig. 4B). These results indicate that, although STAT3 binding to IFNAR1 is not dependent on Tyr^{656} phosphorylation, Tyr^{656} of STAT3 is a site for IFN-dependent tyrosine phosphorylation and is required for p85 interaction with STAT3.

Because PI 3-kinase has endogenous serine kinase activity and activates a serine kinase cascade through the production of PI 3-phosphates (*17*, *18*, *24*, *28*), we examined

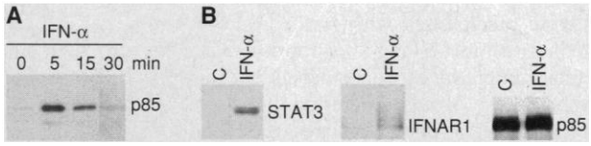

Fig. 1. Coprecipitation of p85 with the IFNAR1 chain from IFN-treated Daudi cells. (**A**) Proteins from control or IFN-α–treated (5000 IU/ml) cell lysates were precipitated with anti-IFNAR1 (*30*). The proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE), blotted onto polyvinylidene difluoride (PVDF) membranes, and probed with anti-p85. (**B**) Lysates were precipitated with anti-p85, resolved by SDS-PAGE, blotted with anti-STAT3, anti-IFNAR1, or anti-p85, and visualized by enhanced chemiluminescence (ECL, Amersham). C, control cells.

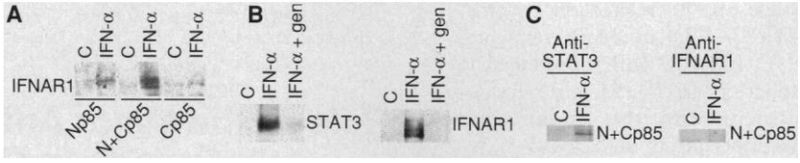

Fig. 2. Precipitation of the tyrosine-phosphorylated IFNAR1 chain and STAT3 in IFN-treated Daudi cells with p85-GST fusion proteins, and direct blotting of STAT3 with the p85-GST fusion protein. (**A**) Lysates from control or IFN-α–treated (5000 IU/ml, 15 min) cells were incubated with Np85, Cp85, or N+Cp85 GST fusion proteins bound to glutathione-agarose beads (*22*). The material bound was resolved by SDS-PAGE, blotted onto PVDF membranes, and probed with anti-IFNAR1 [monoclonal antibody (mAb) 40H2]. (**B**) Proteins from lysates of IFN-treated (5000 IU/ml, 15 min) cells were precipitated with N+Cp85 and resolved by SDS-PAGE, and blots were probed with anti-STAT3 or anti-IFNAR1. Cells were treated in the presence or absence of genistein (gen, 100 μM) for 30 min before addition of IFN-α to test for the role of tyrosine phosphorylation in the interaction of the IFNAR1 chain with p85. (**C**) Proteins from lysates of IFN-treated (5000 IU/ml, 15 min) cells were precipitated with anti-STAT3 or anti-IFNAR1 and resolved by SDS-PAGE. The blots were probed with N+Cp85 and visualized by ECL with the use of a mouse GST mAb and a horseradish peroxidase–conjugated goat antibody to mouse IgG (Southern Biotechnology Associates).

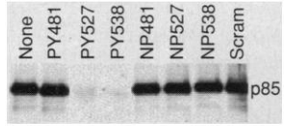

Fig. 3. Effects of phosphopeptides on association of p85 with the IFNAR1 chain. Phosphopeptides (5 μM) corresponding to the amino acids surrounding intracellular tyrosine residues of IFNAR1 [PY481, IDEY(PO_4)FSEQPL; PY527, HKKY(PO_4)SSQTSQ; PY538, SGNY(PO_4)SNEDES; or scram, DHIY(PO_4)LDTSFN] (*19*) or nonphosphorylated peptides (NP481, NP527, or NP538) were added to streptolysin O–permeabilized Daudi cells (*31*). IFN-α–treated (5000 IU/ml, 15 min) cells were assayed for interaction of IFNAR1 with p85 by blotting anti-IFNAR1 precipitates with anti-p85 and were visualized by ECL. For the sample marked "none," IFN-treated cells were permeabilized but no peptide was introduced. Scram represents an irrelevant phosphopeptide.

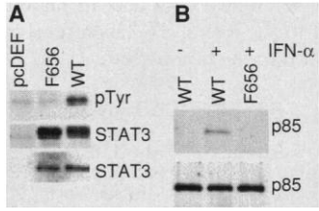

Fig. 4. Effect of wild-type and mutant STAT3 expression on the interaction of IFNAR1 with p85 in COS cells. Wild-type (WT) STAT3 or F656-STAT3 was cloned into the pcDEF1 expression vector (*27*). COS-7 cells were cotransfected by electroporation with the STAT3 and IFNAR1 (in pcDEF1) expression vectors. (**A**) Two days after transfection, IFN-α–treated (5000 IU/ml, 15 min) cell lysates were precipitated with anti-IFNAR1 (lower gel) or anti-STAT3 (upper two gels). Proteins were resolved by SDS-PAGE, blotted onto PVDF membranes, probed with anti-STAT3 or anti-pTyr as indicated, and visualized by ECL. (**B**) Proteins were precipitated with anti-IFNAR1 (upper gel) or anti-p85 (lower gel), probed with anti-p85, and visualized by ECL.

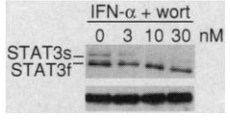

Fig. 5. Effect of wortmannin on STAT3 serine phosphorylation in Daudi cells. Cells were treated with wortmannin (0 to 30 nM) for 30 min before addition of IFN-α. Proteins from lysates of IFN-treated (5000 IU/ml, 15 min) cells were precipitated with anti-pTyr, blotted with anti-STAT3 (upper gel) or anti-TYK2 (lower gel), and visualized by ECL. The faster and slower migrating forms of STAT3 are indicated as STAT3f and STAT3s, respectively.

the effect of the specific PI 3-kinase inhibitor wortmannin on STAT3 phosphorylation (Fig. 5). Cells were pretreated with various concentrations of wortmannin before addition of IFN-α and lysed. Proteins from lysates were precipitated with anti-pTyr and blotted with anti-STAT3 or anti-TYK2. Wortmannin produced a dose-dependent reduction in the slowly migrating band of STAT3, which is phosphorylated on both serine and tyrosine (median inhibitory concentration $IC_{50} \approx 3$ nM). This was not attributable to inhibition of tyrosine phosphorylation events, because wortmannin had little or no effect on phosphorylation of the faster migrating band, which contains STAT3 solely phosphorylated on tyrosine, or of the TYK2 JAK kinase. Moreover, wortmannin (10 nM, 30 min) produced a ~50% reduction in *ISG54* gene induction, consistent with the finding that serine phosphorylation of STAT3 is required for maximal activation of transcription (*16*). These findings indicate a role for the PI 3-kinase pathway in the serine phosphorylation of STAT3. It remains to be established whether PI 3-kinase mediates these events directly or indirectly through the IFN-α–activated PKCs, PKCδ and PKCε (*14*, *17*, *18*, *22*, *28*).

Our results indicate that PI 3-kinase is coupled to the IFN-R through STAT3. Upon ligand-dependent tyrosine phosphorylation, residues Tyr^{527} and Tyr^{538} of IFNAR1 recruit STAT3 to the receptor; then, STAT3 itself undergoes tyrosine phosphorylation at residues Tyr^{656} and Tyr^{705}. Because both SH2 domains of p85 are required for the strongest interaction with the IFNAR1 signaling complex, two tyrosine-phosphorylated STAT3 molecules may be needed to dock p85 efficiently. Once docked, p85 can also undergo IFN-dependent tyrosine phosphorylation. Activated PI 3-kinase can then promote the serine phosphorylation of STAT3, which is critical for the formation of stable STAT3 homodimers or STAT3-STAT1 heterodimers (*16*, *29*). These data add an important new facet to the role of STAT proteins in cellular signal transduction pathways.

REFERENCES AND NOTES

1. A. A. Branca, C. R. Faltynek, S. B. D'Alessandro, C. Baglioni, *J. Biol. Chem.* **257**, 13291 (1982).
2. G. Uze, G. Lutfalla, I. Gresser, *Cell* **60**, 225 (1990).
3. D. Novick, B. Cohen, M. Rubinstein, *ibid.* **77**, 391 (1994).
4. P. Domanski *et al.*, *J. Biol. Chem.* **270**, 21606 (1995).
5. O. R. Colamonici, B. Porterfield, P. Domanski, S. N. Constantinescu, L. M. Pfeffer, *ibid.* **269**, 9598 (1994).
6. S. N. Constantinescu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9602 (1994).
7. O. Colamonici *et al.*, *Mol. Cell. Biol.* **14**, 8133 (1994).
8. R. L. Friedman and G. R. Stark, *Nature* **314**, 637 (1985).
9. A. C. Larner, A. Chaudhuri, J. E. Darnell Jr., *J. Biol. Chem.* **261**, 453 (1986).
10. J. E. Darnell Jr., I. M. Kerr, G. R. Stark, *Science* **264**, 1415 (1994).
11. C. Schindler, K. Shuai, V. R. Prezioso, J. E. Darnell Jr., *ibid.* **257**, 809 (1992).
12. X.-Y. Fu, *Cell* **70**, 323 (1992).
13. L. M. Pfeffer, B. Strulovici, A. R. Saltiel, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6537 (1990).
14. L. M. Pfeffer *et al.*, *ibid.* **88**, 7988 (1991).
15. N. C. Reich and L. M. Pfeffer, *ibid.* **87**, 8761 (1990).
16. Z. Wen, Z. Zhong, J. E. Darnell Jr., *Cell* **82**, 241 (1995).
17. B. M. T. Burgering and P. J. Coffer, *Nature* **376**, 599 (1995).
18. T. F. Franke *et al.*, *Cell* **81**, 727 (1995).
19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. J. E. Mullersman and L. M. Pfeffer, *Trends Biochem. Sci.* **20**, 55 (1994).
21. Z. Songyang *et al.*, *Cell* **72**, 767 (1993).
22. C. H. Yang *et al.*, *J. Biol. Chem.* **271**, 8057 (1996).
23. C. H. Yang and L. M. Pfeffer, unpublished data.
24. R. Kapeller and L. C. Cantley, *Bioessays* **16**, 565 (1994).
25. S. Uddin *et al.*, *J. Biol. Chem.* **270**, 15938 (1995).
26. N. Stahl *et al.*, *Science* **267**, 1349 (1995).
27. L. A. Goldman, E. C. Cutrone, S. V. Kotenko, C. D. Krause, J. A. Langer, *Biotechniques* **21**, 1013 (1996).
28. A. Toker *et al.*, *J. Biol. Chem.* **269**, 32358 (1994).
29. X. Zhang, J. Blenis, H.-C. Li, C. Schindler, S. Chen-Kiang, *Science* **267**, 1990 (1995).
30. S. N. Constantinescu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10487 (1995).
31. H. Yan, K. Krishnan, J. T. E. Lim, L. G. Contillo, J. J. Krolewski, *Mol. Cell. Biol.* **16**, 2074 (1996).

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Role of the Major Antigen of *Mycobacterium tuberculosis* in Cell Wall Biogenesis

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The dominant exported proteins and protective antigens of *Mycobacterium tuberculosis* are a triad of related gene products called the antigen 85 (Ag85) complex. Each has also been implicated in disease pathogenesis through its fibronectin-binding capacities. A carboxylesterase domain was found within the amino acid sequences of Ag85A, B, and C, and each protein acted as a mycolyltransferase involved in the final stages of mycobacterial cell wall assembly, as shown by direct enzyme assay and site-directed mutagenesis. Furthermore, the use of an antagonist (6-azido-6-deoxy-α,α′-trehalose) of this activity demonstrates that these proteins are essential and potential targets for new antimycobacterial drugs.

Mycobacterium tuberculosis possesses a cell wall dominated by covalently linked mycolic acids, D-arabino-D-galactan, and peptidoglycan (mAGP), the mycolic acids of which are complemented by glycolipids such as α,α′-trehalose dimycolate (TDM, cord factor) and α,α′-trehalose monomycolate (TMM) (*1*). This mycolic acid–based permeability barrier shields the organism from environmental stress and contributes to disease persistence and the refractoriness of *M. tuberculosis* to many antibiotics (*1*). The success of chemotherapeutic agents such as isoniazid and ethambutol that specifically inhibit cell wall biogenesis confirms the necessity of this structure for bacterial survival (*2*). The biosynthetic pathways leading to formation of the key mycobacterial cell wall components, arabinogalactan (AG) and mycolic acids, are therefore desirable targets for the rational design of new antituberculosis agents (*3*, *4*). However, there is little information on individual enzymes (*5*, *6*) or genes (*7*) involved in these unique processes.

To define the enzymes and genes responsible for mycolic acid deposition, we developed a mycolyltransferase assay in which nonradioactive mycolic acids from lipid-soluble TMM were transesterified to radioactive water-soluble [^{14}C]α,α′-trehalose, resulting in the formation of lipid-soluble [^{14}C]TMM and [^{14}C]TDM (*6*). The enzyme responsible for this exchange from *M. smegmatis* was purified to near homogeneity by conventional means (*6*), and the transferase activity, assessed in terms of product formation (*8*), was determined to be 1.89×10^4 cpm mg^{-1} protein min^{-1}. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) (*9*) of this enzyme preparation revealed the presence of two major proteins with identical isoelectric points of ~5.1 and relative molecular masses of 31 and 34 kD. Amino acid analysis of the NH_2-terminus of both proteins yielded the sequence RPGLPVEY (*10*). Unexpectedly, this sequence was similar to that reported for

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