supposition in a native ecosystem. The data presented here demonstrating that herbivory by Serengeti grazers tangibly accelerates the mineralization of two minerals of considerable importance in animal nutrition are consistent with simulation results from grassland ecosystem models (*3*). In addition, they indicate that the accelerated recycling of plant-available Na is probably the mechanism leading to levels of that animal nutrient in grazer-exploited Serengeti grasslands that are sufficient to alleviate nutritional shortage in the grazers, particularly reproductive females and growing young (*5*).

Mammalian herbivores have been pervasive in grasslands through evolutionary time (*12*), their levels of forage consumption are considerable (*6*, *13*), and the animals accelerate rather than retard nutrient cycling. The intensity of the plant-herbivore interaction in grasslands, and its evolutionary antiquity, may have attenuated detrimental interaction effects through coevolution (*1*). Overgrazing of grasslands, on the other hand, which is commonly associated with the replacement of free-ranging wild herbivores with livestock and the resulting higher animal densities (*14*), often causes the replacement of highly palatable forages (*15*) that produce easily decomposable litter (*10*) with other plant species of lower nutritional quality and decomposability.

These data provide evidence that a terrestrial grazer can modify ecosystem processes in such a way as to alleviate nutritional deficiencies and, therefore, plausibly to elevate the carrying capacity of the ecosystem. The data also identify accelerated nutrient cycling as an important property of habitats that are critical to large mammal conservation (*16*). The coupling of animal site preference with nutritional effects could provide a guide for identifying sites essential for planning large mammal conservation in natural ecosystems. In addition, the presence of such sites, and the role of mammals in maintaining them, provide clear evidence that habitat deterioration is not an inescapable consequence of increased density of organisms (*1*).

REFERENCES AND NOTES

1. A. F. Hunter and L. W. Aarssen, *BioScience* **38**, 34 (1988); T. S. Bianchi, C. G. Jones, M. Shachak, *Trends Ecol. Evol.* **4**, 234 (1989); M. D. Bertness and R. Callaway, *ibid.* **9**, 191 (1994); C. G. Jones, J. H. Lawton, M. Shachak, *Oikos* **69**, 373 (1994).
2. R. G. Woodmansee, *BioScience* **28**, 448 (1978); S. J. McNaughton, *Oikos* **40**, 329 (1983); J. K. Detling, in *Concepts of Ecosystem Ecology: A Comparative View*, L. R. Pomeroy and J. J. Alberts, Eds. (Springer-Verlag, New York, 1988), pp. 131–148; D. A. Frank *et al.*, *Biogeochemistry* **26**, 163 (1994).
3. E. A. Holland and J. K. Detling, *Ecology* **71**, 1040 (1990); E. A. Holland, W. J. Parton, J. K. Detling, D. L. Coppock, *Am. Nat.* **140**, 685 (1992); S. W. Seagle, S. J. McNaughton, R. W. Ruess, *Ecology* **73**, 1105 (1992); D. A. Frank and R. D. Evans, *ibid.* **78**, 2238 (1997).
4. J. Pastor, R. J. Naiman, B. Dewey, P. F. McInness, *BioScience* **38**, 770 (1988); J. Pastor, B. Dewey, R. J. Naiman, P. F. McInness, Y. Cohen, *Ecology* **74**, 467 (1993).
5. S. J. McNaughton, *Nature* **334**, 343 (1988); *ibid.* **345**, 613 (1990).
6. ———, *Ecol. Monogr.* **53**, 291 (1983); *ibid.* **55**, 259 (1985).
7. Procedures followed R. S. Siegel, *Soil Sci. Soc. Am. J.* **44**, 943 (1980); R. J. Raison, M. J. Connell, P. K. Khanna, *Soil Biol. Biochem.* **19**, 521 (1987); A. Mehlich, *Comm. Soil Sci. Plant Anal.* **15**, 1409 (1984); F. J. Adamsen, D. S. Bigelow, G. R. Scott, *ibid.* **16**, 883 (1985); and (*5*). Soils for N analyses were extracted with 1 M KCl, soils for Na analyses were extracted with Mehlich solution No. 3, and both extracts were preserved with phenylmercuric acetate. Mineral N as NH_4 and NO_3 was determined by continous flow analysis (Lachat QuikChem AE, Milwaukee, WI), and Na was determined by inductively coupled argon plasma spectrometry [Leman Labs PS3000 (Hudson, MA) in simultaneous mode]. Data were analyzed by nested (plot within site) repeated measures of analysis of variance [C. N. von Ende, in *Design and Analysis of Ecological Experiments*, S. M. Scheiner and J. Gurevitch, Eds. (Chapman and Hall, New York, 1993), pp. 113–137].
8. J. K. Detling and E. L. Painter, *Oecologia* **57**, 65 (1983); W. G. Gold and M. M. Caldwell, *ibid.* **81**, 437 (1989).
9. K. Mengel and E. A. Kirkby, *Principles of Plant Nutrition* (International Potash Institute, Bern, Switzerland, 1982), pp. 61–62.
10. D. A. Wedin and D. Tilman, *Oecologia* **84**, 433 (1990); S. Enriquez, C. M. Duarte, K. Sand-Jensen, *ibid.* **94**, 457 (1993); D. A. Wedin and D. Tilman, *Science* **274**, 1720 (1996); J. P. Grime, J. H. C. Cornelissen, K. Thompson, J. G. Hodgson, *Oikos* **77**, 489 (1996).
11. S. J. McNaughton, F. F. Banyikwa, M. M. McNaughton, *Ecosystems*, in press.
12. S. J. McNaughton, in *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions*, P. W. Price, T. M. Lewinsohn, G. W. Fernandes, W. W. Benson, Eds. (Wiley-Interscience, New York, 1991), pp. 509–522.
13. S. J. McNaughton, M. Oesterheld, D. A. Frank, K. J. Williams, *Nature* **341**, 142 (1989); S. J. McNaughton, in *Terrestrial Global Productivity: Past, Present, Future*, J. Roy, B. Schmid, H. A. Mooney, Eds. (Academic Press, San Diego, CA, in press).
14. M. Oesterheld, O. Sala, S. J. McNaughton, *Nature* **356**, 234 (1992).
15. R. F. Noss and A. Y. Cooperider, *Saving Nature's Legacy* (Island Press, Washington, DC, 1994).
16. S. J. McNaughton and F. F. Banyikwa, in *Serengeti II: Dynamics, Management, and Conservation of an Ecosystem*, A. R. E. Sinclair and P. Arcese, Eds. (Univ. of Chicago Press, Chicago, IL, 1995), pp. 49–70.

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Hyaluronan Synthase of Chlorella Virus PBCV-1

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Sequence analysis of the 330-kilobase genome of the virus PBCV-1 that infects a chlorella-like green algae revealed an open reading frame, A98R, with similarity to several hyaluronan synthases. Hyaluronan is an essential polysaccharide found in higher animals as well as in a few pathogenic bacteria. Expression of the *A98R* gene product in *Escherichia coli* indicated that the recombinant protein is an authentic hyaluronan synthase. *A98R* is expressed early in PBCV-1 infection and hyaluronan is produced in infected algae. These results demonstrate that a virus can encode an enzyme capable of synthesizing a carbohydrate polymer and that hyaluronan exists outside of animals and their pathogens.

Hyaluronan or hyaluronic acid (HA), a member of the glycosaminoglycan family that also includes heparin and chondroitan, is a linear polysaccharide composed of alternating β1,4-glucuronic acid (β1,4-GlcA) and β1,3-*N*-acetylglucosamine (β1,3-GlcNAc) groups. Typically the full-length polymer chains are composed of 10^3 to 10^4 monosaccharides (10^6 to 10^7 daltons). HA is an important structural element in the vitreous humor of eye, synovial fluid, and skin of vertebrates (*1*). Furthermore, HA interacts with proteins such as CD44, RHAMM, and fibrinogen, thereby influencing many natural processes such as angiogenesis, cancer, cell motility, wound healing, and cell adhesion (*2*). HA also constitutes the extracellular capsules of certain bacterial pathogens such as group A and C *Streptococcus* and *Pasteurella multocida* type A (*3*, *4*). These capsules act as virulence factors that protect the microbes from phagocytosis and complement during infection (*5*, *6*). Because HA, a component of the host tissues, is not normally immunogenic, the capsule serves as molecular camouflage (*7*).

HA synthases (HASs) are integral mem-

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brane proteins that polymerize the HA molecule using activated uridine diphosphate (UDP)–sugar nucleotides as substrates. Amino acid sequences for some HASs have been deduced from gene sequencing (*8*); their sizes range from 419 to 588 residues. The vertebrate enzymes (DG42, HAS1, HAS2, and HAS3) and streptococcal HasA have several regions of sequence similarity. Recently, while sequencing the double-stranded DNA genome of virus PBCV-1 (*Paramecium bursaria* chlorella virus), we unexpectedly discovered an open reading frame (ORF), A98R (GenBank accession number U42580), encoding a 568-residue protein with similarity to the known HASs (28 to 33% amino acid identity in pairwise comparisons by FASTA) (Fig. 1).

PBCV-1 is the prototype of a family (Phycodnaviridae) of large (175 to 190 nm in diameter) polyhedral, plaque-forming viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae (*9*). PBCV-1 virions contain at least 50 different proteins and a lipid component located inside the outer glycoprotein capsid (*10*). The PBCV-1 genome is a linear, nonpermuted 330-kb double-stranded DNA molecule with covalently closed hairpin ends (*11*).

On the basis of its deduced amino acid sequence, the *A98R* gene product should be an integral membrane protein. To test this hypothesis, we produced recombinant A98R protein in *Escherichia coli* and assayed the membrane fraction for HAS activity (*12, 13*). UDP-GlcA and UDP-GlcNAc were incorporated into polysaccharide by the membrane fraction derived from cells containing the A98R ORF on a plasmid, pCVHAS, (average specific activity of 2.5 pmol of GlcA transferred per minute per microgram of protein), but not by samples from control cells (<0.001 pmol of GlcA transferred per minute per microgram of protein). No activity was detected in the soluble fraction of cells transformed with pCVHAS. UDP-GlcA and UDP-GlcNAc were simultaneously required for polymerization. The activity was optimal in Hepes buffer at pH 7.2 in the presence of 15 mM $MnCl_2$, whereas no activity was detected if the metal ion was omitted. The ions Mg^{2+} and Co^{2+} were ~20% as effective as Mn^{2+} at similar concentrations. The *P. multocida* HAS (*14*) has a similar metal requirement, but other HASs prefer Mg^{2+}.

We also tested the specificity of recombinant A98R for UDP-sugars (*15*). Only the two authentic HA precursors were incorporated into polysaccharide; neither UDP-galacturonic acid (UDP-GalA) nor UDP-*N*-acetylgalactosamine (UDP-GalNAc), the C4 epimers of UDP-GlcA or UDP-GlcNAc, respectively, were incorporated. Likewise, UDP-glucose (UDP-Glc) was not polymerized in place of either HA precursor. This strong substrate specificity for UDP-GlcA and UDP-GlcNAc is a general feature of the HASs HasA (*13*) and DG42 (*16*).

The recombinant A98R enzyme synthesized a polysaccharide with an average molecular size of 3×10^6 to 6×10^6 daltons (Fig. 2), which is smaller than that of the HA synthesized by recombinant HasA or DG42 in vitro (~10^7 daltons and ~5×10^6 to 8×10^6 daltons, respectively) (*13, 16*).

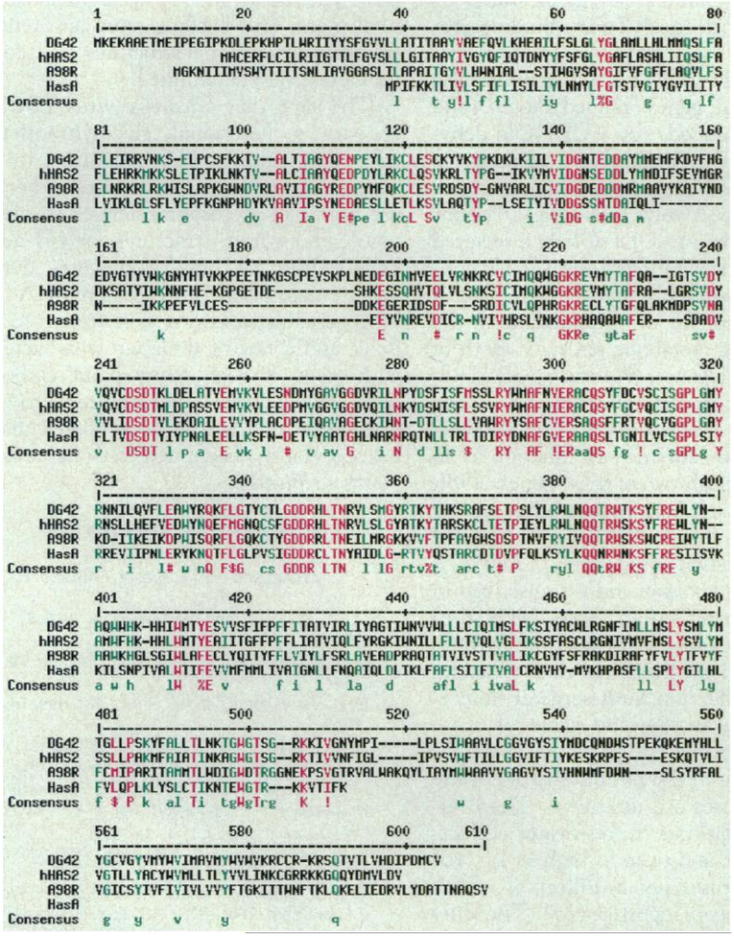

Fig. 1. Sequence similarity of HASs. The Multalin program (*26*) was used to align the amino acid sequences of HASs *Xenopus laevis* DG42, human HAS2, PBCV-1 A98R, and *Streptococcus pyogenes* HasA (red, 90% consensus; green, 50% consensus, as calculated by Multalin) (*8*). In the consensus sequence, the symbols are: !, any one of I or V; $, any one of L or M; %, any one of F or Y; #, any one of N,D,E, or Q. Single-letter 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.

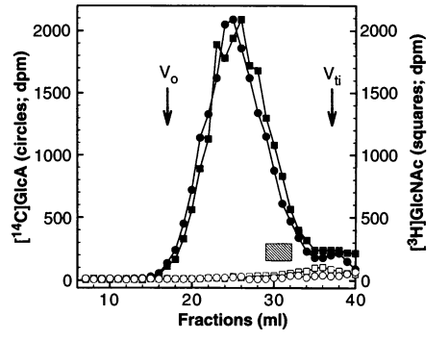

Fig. 2. Size exclusion chromatography of polymer product of recombinant A98R HAS. Membranes derived from *E. coli* cells transformed with pCVHAS were incubated with both radiolabeled HA precursors diluted to the same specific activity (*27*). After deproteinization and removal of unincorporated precursors, samples were injected onto a Sephacryl S-500HR size exclusion column, and the radioactivity in the fractions was measured (^{3}H, solid squares; ^{14}C, solid circles). A duplicate sample was treated with HA lyase before deproteinization and chromatography (^{3}H, open squares; ^{14}C, open circles); no polymer remains after digestion. Size standards: V_o arrow, void volume, HA derived from recombinant streptococcal HasA (17 ml; ≥2×10^7 daltons) (*13*); crosshatched box, blue dextran (29 to 32 ml; average molecular size 2×10^6 daltons; Pharmacia); V_{ti} arrow, totally included volume, UDP-sugars (37 ml).

The polysaccharide was completely degraded by *Streptomyces hyalurolyticus* HA lyase, an enzyme that depolymerizes HA but not structurally related glycosaminoglycans such as heparin and chondroitan (*17*).

We examined PBCV-1–infected chlorella cells for *A98R* gene expression. A ~1700-nucleotide *A98R* transcript appeared about 15 min after infection and disappeared by 60 min after infection (*18*), indicating that *A98R* is an early gene. Consequently, we assayed membrane fractions from uninfected and PBCV-1–infected chlorella cells at 50 and 90 min after infection for HAS activity. Infected cells, but not uninfected cells, had activity (Table 1). Like the bacterially derived recombinant A98R enzyme, radioactive label incorporation from UDP-[^{14}C]GlcA into polysaccharide depended on both Mn^{2+} and UDP-GlcNAc. This labeled product was also degraded by HA lyase. Disrupted PBCV-1 virions had no HAS activity.

PBCV-1–infected chlorella cells were analyzed for HA polysaccharide by means of a highly specific ^{125}I-labeled HA-binding protein (*19*, *20*). Extracts from cells at 50 and 90 min after infection contained substantial amounts of HA (0.7 and 1400 ng per microgram of protein, respectively), but not extracts from uninfected algae (<0.04 ng per microgram of protein) or disrupted PBCV-1 virions (<0.04 ng per microgram of dry weight). The labeled HA-binding protein also interacted with intact infected cells at 50 and 90 min after infection, but not with healthy cells (*21*). Therefore, a considerable portion of the newly synthesized HA polysaccharide was immobilized at the outer cell surface of the infected algae.

Table 1. HAS activity of membranes derived from *Chlorella* cells infected with PBCV-1. The membrane fractions (370 μg of protein) from uninfected cells or cells at 50 and 90 min after infection (a.i.) were assayed with UDP-[^{14}C]GlcA (60 μM, 0.02 μCi) in parallel reactions containing the following components as indicated (300 μM UDP-GlcNAc or 15 mM $MnCl_2$ or both) for 1 hour at 30°C (*28*). HAS specific activity (presented as picomoles of [^{14}C]GlcA transferred per hour per milligram of protein) was detected in the algal membranes after infection with PBCV-1, but not in uninfected cells.

Sample	UDP-GlcNAc	Mn^{2+}	HAS specific activity
Uninfected	+	+	≤6
	+	−	≤6
	−	+	≤6
50 min a.i.	+	+	42
	+	−	≤6
	−	+	≤6
90 min a.i.	+	+	170
	+	−	≤6
	−	+	≤6

The extracellular HA does not play any obvious role in the interaction between the virus and its algal host because neither plaque size nor plaque number was altered by including either testicular hyaluronidase (465 units/ml) or free HA polysaccharide (100 μg/ml) in the top agar of the PBCV-1 plaque assay (*9*).

Among chlorella viruses, HA biosynthesis during infection is not limited to the PBCV-1 prototype strain. Thirty-three independently isolated and plaque-purified viruses from the United States, South America, Asia, and Australia were tested for the presence of an A98R-like gene and for the ability to direct production of HA polysaccharide in *Chlorella* NC64A. Dot-blot hybridization analyses of the individual viral genomes with the PBCV-1 A98R probe indicated that 19 isolates (58%) had a similar gene; the algal host DNA did not cross-react with the probe (*21*). Chlorella cells infected with each of these 19 viruses produced cell surface HA as measured by interaction with the ^{125}I-HA–binding protein (*21*).

Surprisingly, the PBCV-1 genome also has additional genes, named *A609L* and *A100R*, that encode for a UDP-Glc dehydrogenase (UDP-Glc DH) and a glutamine: fructose-6-phosphate amidotransferase (GFAT), respectively. UDP-Glc DH converts UDP-Glc into UDP-GlcA, a required precursor for HA biosynthesis. GFAT converts fructose-6-phosphate into glucosamine-6-phosphate, an intermediate in the UDP-GlcNAc metabolic pathway. Both of these PBCV-1 genes, like the *A98R* HAS, are expressed early in infection and encode enzymatically active proteins (*22*); however, these three genes do not function as an operon. Although two of these genes, *A98R* and *A100R*, are near one another in the viral genome (bases 50,901 to 52,607 and 52,706 to 54,493, respectively), *A609L* is located ~240 kb away and is transcribed in the opposite orientation (bases 292,916 to 291,747). The presence of multiple enzymes in the HA biosynthesis pathway indicates that HA production must serve an important function in the life cycle of these chlorella viruses.

The details of the natural history of the phycodnaviruses are unknown. These viruses are ubiquitous in freshwater collected worldwide, and titers as high as 4×10^4 infectious viruses per milliliter of native water have been reported (*23*). The only known hosts for these viruses are chlorella-like green algae, which normally live as hereditary endosymbionts in some isolates of the ciliate, *P. bursaria*. In the symbiotic unit, algae are enclosed individually in perialgal vacuoles and are surrounded by a host-derived membrane (*24*). The endosymbiotic chlorella are resistant to virus infection and are only infected when they are outside the paramecium (*9*). We hypothesize that HA synthesis and its accumulation on the algal surface may block the uptake of virus-infected algae by the paramecium. Alternatively, the chlorella viruses might have another host in nature (such as an aquatic animal); perhaps the virus is transmitted because this other host is attracted to or binds to the HA polysaccharide on virus-infected algae.

As depicted in Fig. 1, HASs of *Streptococcus*, vertebrates, and PBCV-1 have many motifs of two to four residues that occur in the same relative order. These conserved motifs probably reflect domains crucial for HA biosynthesis. Regions of similarity between HASs and other enzymes that synthesize β-linked polysaccharides from UDP-sugar precursors are also being discovered as more glycosyltransferases are sequenced (*25*). The significance of these similar structural motifs will become more apparent as the three-dimensional structures of glycosyltransferases are determined.

The fact that *Chlorella* virus PBCV-1 encodes a functional glycosyltransferase that can synthesize HA is contrary to the general observation that viruses either (i) use host cell glycosyltransferases to create new carbohydrate structures, or (ii) accumulate host cell glycoconjugates during virion maturation. Furthermore, HA has been generally regarded as restricted to animals and a few of their virulent bacterial pathogens. Though many plant carbohydrates have been characterized, to our knowledge, neither HA nor a related analog has previously been detected in cells of plants or protists.

REFERENCES AND NOTES

1. T. C. Laurent and J. R. E. Fraser, *FASEB J.* **6**, 2397 (1992).
2. C. B. Knudson and W. Knudson, *ibid.* **7**, 1234 (1993).
3. E. H. Kass and C. V. Seastone, *J. Exp. Med.* **79**, 319 (1944).
4. G. R. Carter and E. Annau, *Am. J. Vet. Res.* **14**, 475 (1953).
5. B. G. Harmon, J. R. Glisson, K. S. Latimer, W. L. Steffens, J. C. Nunnally, *ibid.* **52**, 1507 (1991).
6. M. R. Wessels, A. E. Moses, J. B. Goldberg, T. J. DiCesare, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8317 (1991).
7. A. W. Quinn and K. P. Sing, *Proc. Soc. Exp. Biol. Med.* **95**, 290 (1957).
8. P. L. DeAngelis, J. Papaconstantinou, P. H. Weigel, *J. Biol. Chem.* **268**, 19181 (1993); P. H. Weigel, V. C. Hascall, M. Tammi, *ibid.* **272**, 13997 (1997).
9. J. L. Van Etten, L. C. Lane, R. H. Meints, *Microbiol. Rev.* **55**, 586 (1991); J. L. Van Etten, *Mol. Cells* **5**, 99 (1995); ———, D. E. Burbank, D. Kuczmarski, R. H. Meints, *Science* **219**, 994 (1983).
10. M. P. Skrdla, D. E. Burbank, Y. Xia, R. H. Meints, J. L. Van Etten, *Virology* **135**, 308 (1984); I.-N. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3840 (1993).
11. J. Rohozinski, L. E. Girton, J. L. Van Etten, *Virology*

168, 363 (1989); Y. Zhang, P. Strasser, R. Grabherr, J. L. Van Etten, *ibid.* **202**, 1079 (1994).
12. The A98R ORF was cloned after amplification of genomic viral DNA with 22 cycles of polymerase chain reaction (PCR) with Taq polymerase [F. M. Ausubel *et al.*, in *Short Protocols in Molecular Biology* (Wiley, New York, ed. 3, 1995)]. The oligonucleotide primers [sense 5′-gagagctccATGGGTAAAAA-CATTATCATAATGG-3′; antisense 5′-gcatgtcgacT-CACACAGACTGAGCATTGGTAG-3′; Great American Gene Company] contained Nco I or Sal I restriction sites (underlined, respectively) flanking the ORF encoding 568 amino acids (uppercase letters). Codons 4 and 5 were altered to optimize bacterial expression. The PCR product was purified, digested with Nco I and Sal I, and ligated into a modified version of the plasmid pET-8C [B. A. Moffatt and F. W. Studier, *J. Mol. Biol.* **189**, 113 (1986)] (it has an extra Sal I site in the polylinker) cleaved with Nco I and partially digested with Sal I. This construct placed the A98R ORF under the control of a T7 phage promoter. The resulting plasmid, pCVHAS, was transformed into the expression host, *E. coli* BL21(DE3). The A98R protein was expressed by induction with 1 mM isopropylthiogalactoside. After 3 to 5 hours of further growth, the membrane fraction was isolated (*13*). Control membrane preparations were made from cultures with the same vector containing an irrelevant gene (a protein kinase). Total protein was measured according to M. M. Bradford [*Anal. Biochem.* **72**, 248 (1976)]. The paper chromatography method was used to assay for HAS activity (*13*).
13. P. L. DeAngelis and P. H. Weigel, *Biochemistry* **33**, 9033 (1994).
14. P. L. DeAngelis, *ibid.* **35**, 9768 (1996).
15. Assays with *E. coli*–derived A98R were incubated either with 120 μM UDP-[^{14}C]GlcA or with 150 μM UDP-[^{3}H]GlcNAc. The reactions also contained one unlabeled sugar nucleotide (an authentic precursor or UDP-Glc, UDP-GalA, or UDP-GalNAc) at 300 μM. Less than 5% of the maximal incorporation (assay with UDP-GlcA and UDP-GlcNAc present) was detected if an unnatural UDP-sugar was substituted for UDP-GlcA or UDP-GlcNAc, or if only a single precursor was present.
16. P. E. Pummill, A. M. Achyuthan, P. L. DeAngelis, in preparation.
17. T. Ohya and Y. Kaneko, *Biochim. Biophys. Acta* **198**, 607 (1970).
18. M. V. Graves, D. Landstein, J. L. Van Etten, unpublished results.
19. A. Tengblad, *Biochem. J.* **185**, 101 (1980).
20. A competitive radiometric assay based on a ^{125}I-labeled HA-binding protein (*19*) (Pharmacia HA Test) was used to measure the amount of HA in disrupted (freeze-thawed) virus particles or the cultures of NC64A cells. The cells were disrupted by vigorous agitation with glass beads (1-mm beads, agitated for 3 min four times; Biospec Mini-Beadbeater-8). The cell lysate was clarified by centrifugation (15,000*g*, 5 min) before assaying (average of two determinations).
21. P. L. DeAngelis, M. V. Graves, J. L. Van Etten, unpublished results.
22. D. Landstein, P. L. DeAngelis, J. L. Van Etten, in preparation.
23. J. L. Van Etten, D. E. Burbank, A. M. Schuster, R. H. Meints, *Virology* **140**, 135 (1985); T. Yamada, T. Higashiyama, T. Fukuda, *Appl. Environ. Microbiol.* **57**, 3433 (1991).
24. W. Reisser, Ed., *Algae and Symbioses* (Biopress, Bristol, UK, 1992).
25. P. L. DeAngelis, N. Yang, P. H. Weigel, *Biochem. Biophys. Res. Commun.* **199**, 1 (1994); I. M. Saxena *et al.*, *J. Bacteriology* **177**, 1419 (1995).
26. F. Corpet, *Nucleic Acids Res.* **16**, 10881 (1988).
27. Membranes (860 μg of protein) were incubated with 120 μM UDP-[^{14}C]GlcA (0.36 μCi) and 840 μM UDP-[^{3}H]GlcNAc (2.6 μCi) in 300 μl of 50 mM Hepes, pH 7.2, with 15 mM $MnCl_2$ for 3 hours at 30°C. EDTA (18 mM final concentration) was then added to stop the HAS activity. Half of the reaction was deproteinized by treatment with 0.5% SDS (w/v) and Pronase (final concentration of 200 μg/ml, 5 hours at 37°C; Boehringer Mannheim). Unincorporated precursors and other small molecules were removed by ultrafiltration (Microcon10, 10^4-daltons cutoff; Amicon). Half of this semipurified sample was injected onto a Sephacryl S-500HR column (1 cm by 50 cm; Pharmacia) equilibrated in 0.2 M NaCl, 5 mM tris, pH 8 (0.5 ml/min, 1-ml fractions). To verify that the identity of the labeled polysaccharide was HA, we treated the other half of the original reaction with HA lyase (30 units at 37°C overnight; Sigma) before the deproteinization step. This treatment degraded the radioactive polymer to small oligosaccharides (tetramers and hexamers) that were removed by ultrafiltration before gel filtration chromatography.
28. Two cultures of NC64A cells (0.9 liter; 1.9×10^{10} cells) were infected with PBCV-1 (multiplicity of infection of 5) and incubated for 50 or 90 min after infection. Another culture served as an uninfected control. The cells were harvested, and the membrane fraction (yield ~3 mg of protein) was prepared as described [P. L. DeAngelis and A. M. Achyuthan, *J. Biol. Chem.* **271**, 23657 (1996)], except that 1 mM mercaptoethanol was substituted for dithiothreitol. The paper chromatography method was used to assay for HAS activity (*13*).
29. We thank A. M. Achyuthan, G. M. Air, M. K. Brakke, R. D. Cummings, L. C. Lane, M. Nelson, and P. H. Weigel for helpful discussions. R. A. Steinberg provided the plasmids and host strain for T7 expression system. Supported by a NIH grant (R01-GM56497) and a University of Oklahoma Medical Alumni Scholarship to P.L.D. and a NIH grant (R01-GM32441) to J.V.E.

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Specific Inhibition of Stat3 Signal Transduction by PIAS3

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The signal transducer and activator of transcription–3 (Stat3) protein is activated by the interleukin 6 (IL-6) family of cytokines, epidermal growth factor, and leptin. A protein named PIAS3 (protein inhibitor of activated STAT) that binds to Stat3 was isolated and characterized. The association of PIAS3 with Stat3 in vivo was only observed in cells stimulated with ligands that cause the activation of Stat3. PIAS3 blocked the DNA-binding activity of Stat3 and inhibited Stat3-mediated gene activation. Although Stat1 is also phosphorylated in response to IL-6, PIAS3 did not interact with Stat1 or affect its DNA-binding or transcriptional activity. The results indicate that PIAS3 is a specific inhibitor of Stat3.

Stat3 participates in signal transduction pathways activated by the IL-6 family of cytokines and by epidermal growth factor (*1*, *2*). Stat3 is also activated in cells treated with leptin, a growth hormone that functions in regulating food intake and energy expenditure (*3*). Targeted disruption of the mouse gene encoding Stat3 leads to early embryonic lethality (*4*). Like other members of the STAT family, Stat3 becomes tyrosine phosphorylated by Janus kinases (JAKs). Phosphorylated Stat3 then forms a dimer and translocates into the nucleus to activate specific genes (*5*).

We cloned a protein named PIAS1, which can specifically interact with Stat1 (another member of the STAT family), by the yeast two-hybrid assays (*6*). We searched the expressed sequence tag (EST) database for other PIAS family members and identified a human EST clone encoding a polypeptide related to the COOH-terminal portion of PIAS1 (*7*). We obtained a full-length cDNA containing an open reading frame of 583 amino acids by screening a mouse thymus library with the human EST clone (*8*). The corresponding protein, named PIAS3, contains a putative zinc-binding motif [C_2-$(X)_{21}$-C_2] (*9*), a feature conserved in the PIAS family (Fig. 1A). Northern (RNA) blot analysis indicated that PIAS3 is widely expressed in various human tissues (Fig. 1B).

To study the function of PIAS3, we prepared a specific antiserum (anti-PIAS3c) to a recombinant fusion protein of glutathione-S-transferase (GST) with the 79 COOH-terminal amino acid residues of PIAS3. This antibody detected a protein with a molecular mass of about 68 kD, the predicted size of PIAS3, in both cytoplasmic and nuclear extracts of a number of human and murine cell lines (*10*). To identify which STAT protein interacts with PIAS3, we prepared protein

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