A Protein Kinase Substrate Identified by the Two-Hybrid System

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A genetic method, the two-hybrid system, was used to identify four genes encoding proteins that interact with the SNF1 protein kinase from yeast. One of the genes, *SIP1*, was independently isolated as a multicopy suppressor of defects caused by reduced SNF1 kinase activity, and genetic evidence supports its function in the SNF1 pathway. The SIP1 protein co-immunoprecipitated with SNF1 and was phosphorylated in vitro. Thus, the two-hybrid system, which is applicable to any cloned gene, can be used to detect physical interactions between protein kinases and functionally related substrate proteins.

Protein kinases are key components of many signaling pathways in eukaryotic cells. Understanding the regulatory roles of protein kinases requires the identification of substrates and regulatory proteins, which has often proved difficult. A genetic method for detecting protein interactions in vivo called the two-hybrid system (*1*, *2*) offers an approach to this problem. The method detects the reconstitution of function of GAL4, a transcriptional activator from yeast. The interaction of two hybrid proteins, one bearing the GAL4 DNA binding domain and the other bearing a transcriptional activation domain, results in activation of a *GAL1-lacZ* reporter gene (Fig. 1).

We have used the two-hybrid system to identify substrates and other proteins that interact with the SNF1 protein kinase in the yeast *Saccharomyces cerevisiae*. The SNF1 Ser-Thr kinase is required for the release of genes from glucose repression (*3*) and also affects other aspects of cell growth control (*4*). A plant homolog of the *SNF1* gene has been cloned, indicating that *SNF1* has been conserved through evolution (*5*). SNF1 is physically associated with the SNF4 protein, which stimulates kinase activity (*6*, *7*). The interaction of SNF1 and SNF4 provided the prototype for the two-hybrid system (*1*).

To identify genes encoding other proteins that interact with SNF1, we screened (*8*) plasmid libraries of fusions between the GAL4 activation domain (residues 768 to 881) and yeast genomic DNA fragments (*2*) for interaction with the DNA binding domain hybrid GAL4(1-147)-SNF1 (*1*) in a yeast reporter strain carrying *GAL1-lacZ*. We assayed a total of 300,000 transformants for β-galactosidase (β-gal) expression by replicating them on nitrocellulose filters and incubating the colonies with the chromogenic substrate X-Gal. From 144 blue colonies, we recovered 16 fusion plasmids that conferred blue color to the reporter strain only in the presence of GAL4(1-147)-SNF1, consistent with the idea that interaction of the new fusion protein with the

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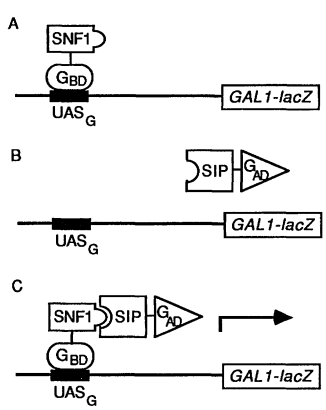

Fig. 1. The two-hybrid system. The interaction between SNF1 and SIP associates the GAL4 activation domain (G_{AD}; residues 768 to 881) with the GAL4 DNA binding domain (G_{BD}; residues 1 to 147), resulting in transcriptional activation of the *GAL1-lacZ* reporter gene. UAS_G, upstream activation site recognized by GAL4. Yeast reporter strains expressed (**A**) GAL4(1-147)-SNF1, (**B**) G_{AD}-SIP, and (**C**) GAL4(1-147)-SNF1 and G_{AD}-SIP.

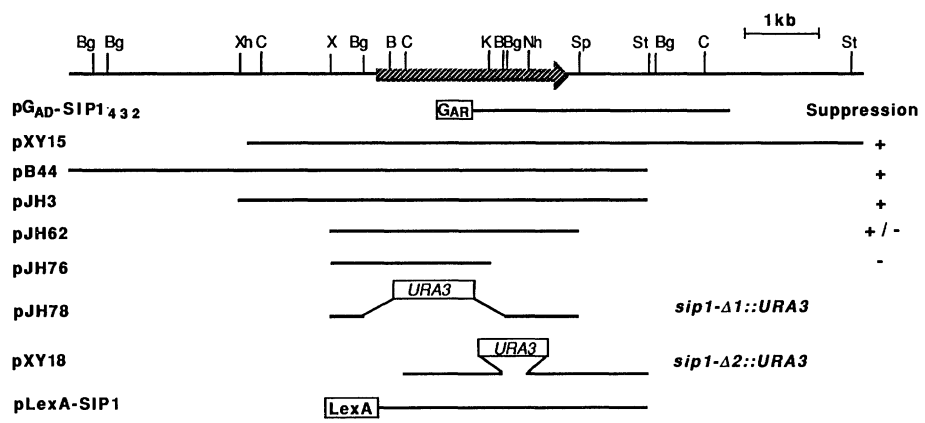

Fig. 2. Maps of *SIP1* plasmids. Heavy arrow, *SIP1* coding region. Vectors were YEp24, except that pJH78 and pXY18 were in pUC19. We manipulated DNA by standard methods (*14*). We constructed pLexA-SIP1 by adding two primers (to direct synthesis of the NH_2-terminus of *SIP1*) to the polymerase chain reaction with a pJH3 derivative as template. The primer 5′-GGGGAATTCACAATGGTAGATATA incorporated an Eco RI site (underlined) 5′ to the initiating ATG codon, and the other primer was complementary to nucleotides 520 through 503. The amplified DNA was digested with Eco RI and Cla I and ligated to the Cla I–Sal I fragment of pJH3 (Sal I in the vector) and the Eco RI–Sal I fragment of LexA(1-202) + PL (*15*). Suppression of the *snf4* raffinose growth defect by multicopy plasmids is indicated. Restriction sites: B, Bam HI; Bg, Bgl II; C, Cla I; K, Kpn I; Nh, Nhe I; Sp, Sph I; St, Stu I; X, Xba I; and Xh, Xho I. Not all Kpn I, Nhe I, or Stu I sites were mapped.

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   1  MVDISDTSGY LHKQGVLPSV SFICTSFFAC GLTSWKVELS YYIVVAAAMG NSPSTQDPSH STKKEHGHHF
  71  HDAFNKDRQG SITSQLFNNR KSTHKRRASH TSEHNGAIPP RMQLLASHDP STDCDGRMSS DTTIDKGPSH
 141  LFKKDYSLSS AADVNDTTLA NLTLSDDHDV GAPEEQVKSP SFLSPGPSMA TVKRTKSDLD DLSTLNYTMV
 211  DETTENERND KPHHERHRSS IIALKKNLLE SSATASPSPT RSSSVHSASL PALTKTDSID IPVRQPYSKK
 281  PSIHAYQYQY LNNDETFSEN SQMDKEGNSD SVDAEAGVLQ SEDMVLNQSL LQNALKKDMQ RLSRVNSSNS
 351  MYTAERISHA NNNGNIENNT RNKGNAGGSN DDFTAPISAT AKMMMKLYGD KTLMERDLNK HHNKTKKAQN
 421  KKIRSVSNSR RSSFASLHSL QSRKSILTNG LNLQPLHPLH PIINDNESQY SAPQHREISH HSNSMSSMSS
 491  ISSTNSTENT LVVLKWKDDG TVAATTEVFI VSTDIASALK EQRELTLDEN ASLDSEKQLN PRIRMVYDDV
 561  HKEWFVPDLF LPAGIYRLQF SINGILTHSN FLPTATDSEG NFVNWFEVLP GYHTIEPFRN EADIDSQVEP
 631  TLDEELPKRP ELKRFPSSSR KSSYYSAKGV ERPSTPFSDY RGLSRSSSIN MRDSFVRLKA SSLDLMAEVK
 701  PERLVYSNEI PNLFNIGDGS TISVKGDSDD VHPQEPPSFT HRVVDCNQDD LFATLQQGGN IDAETAEAVF
 771  LSRYPVPDLP IYLNSSYLNR ILNQSNQNSE SHERDEGAIN HIIPHVNLNH LLTSSIRDEI ISVACTTRYE
 841  GKFITQVVYA PCYYKTQKSQ ISN
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Fig. 3. Predicted amino acid sequence of the *SIP1* product. Nucleotide sequence from positions −122 to +3157 was determined on both strands (*16*), and the GenBank accession number is M90531. Reverse contrast letters mark G_{AD}-SIP1 fusion points. Serine-rich regions containing basic and aliphatic residues begin at positions 241, 424, 478, 647, and 671. 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.

SNF1 hybrid reconstitutes GAL4 function.

The 16 plasmids represent four different genes as judged by colony hybridization. The sequence adjacent to the fusion site in each plasmid was determined by extending a primer from the *GAL4* region, and none matched sequences in the GenBank database. *SNF4* was not recovered; however, fusions at the sites available in *SNF4* lack the NH_2-terminal one-third of the protein. The genes were designated *SIP1* to *SIP4* [SNF1 interacting protein (SIP)]. Ten plasmids contained *SIP1* sequences, and two different fusion points were represented.

To confirm that SIP1 interacts with SNF1, we assayed β-gal expression in the yeast reporter strain carrying hybrid plasmids (Table 1). In combination with GAL4(1-147)-SNF1, each of the two GAL4 activation domain (G_{AD}-SIP1) fusions increased β-gal expression. Moreover, expression was completely dependent on the presence of SNF1 sequences in the DNA binding partner. Thus, SIP1 meets the criteria of the two-hybrid assay for a protein that potentially interacts in vivo with SNF1. We recovered the entire *SIP1* gene, on pXY15 (Fig. 2), from a yeast genomic library (9) by screening with the inserted sequence from pG_{AD}-$SIP1_{432}$ as a probe.

SIP1 is identical to a gene isolated independently on the basis of its functional relation with the SNF1 pathway. We screened for multicopy plasmids that suppress growth defects of an *snf4* mutant, which has impaired SNF1 protein kinase activity (*10*). Out of nine plasmids that restored growth of an *snf4* deletion mutant (*snf4Δ2*) (*7*) on raffinose, pB44 was the most effective. Analysis of subclones derived from pB44 showed that increased dosage of the *SIP1* gene caused the suppression (Fig. 2). Thus, two distinct genetic approaches identified the *SIP1* gene.

Further analysis of the suppression phenotype supported the view that SIP1 is functionally related to SNF1. First, increased *SIP1* gene dosage restored expression of invertase in *snf4Δ* mutants (Table 2), indicating that growth on raffinose reflects restored invertase gene regulation. Second, multiple copies of *SIP1* remedied the pleiotropic defects of an *snf4Δ* mutant, improving both galactose growth and sporulation. Finally, pB44 did not restore growth on raffinose or derepression of invertase in an *snf1* deletion mutant (*snf1Δ*) (Table 2). Thus, increased dosage of *SIP1* does not compensate for loss of the SNF1 protein kinase, but only for the loss of the stimulatory effect of the SNF4 protein. Taken together, this genetic evidence indicates that the *SIP1* product functions in the SNF1 pathway.

To determine the *sip1* mutant phenotype, we constructed (*11*) diploid strains (*ura3/ura3*) heterozygous for the *sip1-Δ1::URA3* or *sip1-Δ2::URA3* mutation (Fig. 2). The diploids were sporulated and subjected to tetrad analysis. The *URA3* marker segregated 2:2, but no growth defect on glucose or raffinose was detected, and *sip1-Δ1::URA3* segregants showed wild-type, regulated invertase expression. The *sip1Δ/sip1Δ* diploids sporulated. Because increased dosage of *SIP1* suppresses *snf4*, we also examined the interaction of *sip1-Δ1* and *snf4-Δ2*. Tetrad analysis of a diploid heterozygous for both mutations revealed a more severe raffinose growth defect for the *sip1Δsnf4Δ* double mutants than for the *snf4Δ* segregants. At 23°C, a semipermissive temperature for *snf4Δ* mutants (*7*), invertase activity in the double mutants was one-half of that in *snf4Δ* mutants (Table 2). Thus, the *sip1Δ* mutation exacerbates the *snf4Δ* mutant phenotype. The minimal effects of *sip1Δ* may reflect the presence of a functionally redundant gene.

The primary structure of the SIP1 protein was deduced from the sequence of a 3.3-kb region containing an open reading frame of 863 codons (Fig. 3). Serine and Thr constitute 20% of the amino acids in the predicted 96-kD protein, and there are several clusters of Ser residues. No substantial similarity to SNF4 or any sequence in the GenBank database (December 1991 release) was found. The region COOH-terminal to codon 432 is sufficient for interaction with SNF1.

Immune complex assays of the SNF1 protein kinase activity demonstrated phosphorylation in vitro of SNF1 and several

Table 1. Transcriptional activation by GAL4 hybrid proteins. Transformants of GGY::171 were grown to mid-logarithmic phase in synthetic complete medium containing galactose (2%), ethanol (2%), and glycerol (2%) and lacking Leu or His or both to select for the plasmids. GAL4, GAL4-SNF1, and SNF4-GAL4 plasmids were pMA424 (*12*), pEE5, and pNI12 (*1*), respectively.

DNA binding hybrid	Activation hybrid	Colony color	β-Gal activity*
GAL4(1-147)-SNF1	—	White	<1
GAL4(1-147)-SNF1	G_{AD}-$SIP1_{201}$	Blue	220
GAL4(1-147)-SNF1	G_{AD}-$SIP1_{432}$	Blue	230
GAL4(1-147)	G_{AD}-$SIP1_{432}$	White	<1
—	G_{AD}-$SIP1_{432}$	White	<1
GAL4(1-147)-SNF1	SNF4-GAL4(768-881)	Blue	38

*Expressed in Miller units (*13*). Values >1 are averages from six transformants with standard errors <12%, and values <1 are from three transformants.

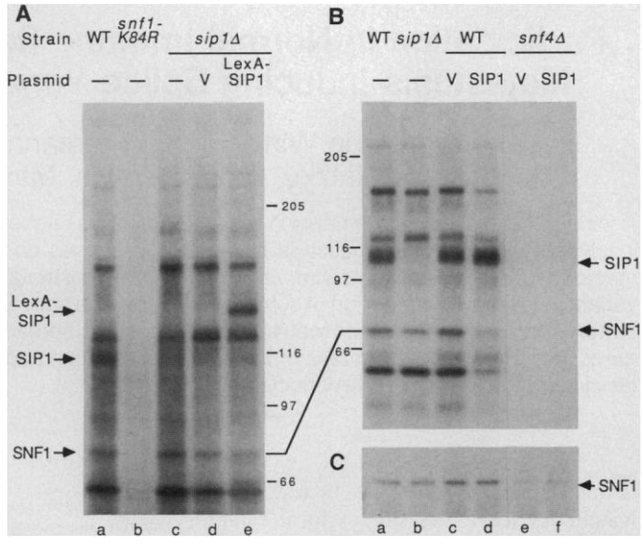

Fig. 4. Immune complex assays of the SNF1 kinase activity. (**A** and **B**) Immune complex kinase assays were as described (*6*) except that samples were stored only briefly at −70°C because detection of several bands was sensitive to storage. Proteins were precipitated with anti-SNF1 (A) or affinity-purified antibody (B), were incubated in buffer containing [γ-^{32}P]-labeled ATP (3000 Ci/mmol; Dupont, NEN Research Products, Boston, Massachusetts), and were separated by SDS–polyacrylamide gel electrophoresis in 6% (A) or 7.5% (B) polyacrylamide. Phosphorylated products were detected by autoradiography. The SIP1 protein was faintly detected in lane f. (B) shows less background labeling because affinity-purified antibody was used. (**C**) Samples of immunoprecipitated protein were analyzed by protein immunoblotting; less SNF1 protein was present in *snf4* mutants than in wild-type strains (*6*). Similar analysis for samples in (A) showed equal amounts of SNF1 and SNF1-K84R proteins. (A) Relevant strain genotypes: a, wild type (WT); b, *snf1-K84R*; c, d, and e, *sip1-Δ1::URA3*. Plasmids: d, vector (V) LexA(1-202)+PL (*15*); e, pLexA-SIP1. (B and C) Strain genotypes: a, c, and d, wild type; b, *sip1-Δ1::URA3*; e and f, *snf4-Δ2*. Plasmids: c and e, vector YEp24; d and f, pJH3. Molecular size standards are in kilodaltons.

Table 2. Effects of *SIP1* gene dosage and mutations on invertase expression. Invertase activity is expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are averages from two to four strains (four for *snf4* strains). For values >1, standard errors were <25%.

Relevant genotype	Plasmid-borne gene	Temperature (°C)	Invertase activity	
			R	D
snf4Δ (YEp24)	None	30°	<1	4*
snf4Δ (pB44)	*SIP1*	30°	<1	37*
snf4Δ (pLN132)	*SNF4*	30°	<1	140*
snf1Δ (pB44)	*SIP1*	30°	<1	<1*
sip1Δ		23°	1	190†
snf4Δ		23°	<1	27†
sip1Δsnf4Δ		23°	<1	12†

*Cultures were grown at 30°C in synthetic complete Ura with 2% glucose (repressing conditions, R) and shifted to 0.05% glucose for derepression (D) (*6*). †Cultures were grown and derepressed in rich medium at 23°C, a semipermissive temperature for *snf4Δ* mutants (*7*).

coprecipitating proteins, and assays of a mutant altered in the conserved Lys of the SNF1 adenosine triphosphate (ATP) binding site (*snf1-K84R*) showed that the phosphorylation is dependent on SNF1 activity (6) (Fig. 4A). One of the phosphorylated products detected in an assay of a wild-type extract migrates as a 110-kD protein, which is approximately the size predicted for SIP1. This phosphoprotein was not detected in assays of an *sip1Δ* mutant (Fig. 4, A and B), suggesting that this protein corresponds to SIP1. We constructed a plasmid expressing a LexA-SIP1 fusion protein (Fig. 2), which was detected as a 130-kD protein on protein immunoblots. In an assay of an *sip1Δ* mutant carrying this plasmid, a new phosphoprotein corresponding to LexA-SIP1 was detected (Fig. 4A). These results show that the SIP1 protein co-immunoprecipitates with SNF1 and is phosphorylated in vitro. The diffuse nature of the band suggests that SIP1 is heterogeneously modified. The absence of phosphorylation in immune complexes containing the SNF1-K84R mutant protein indicates that phosphorylation of SIP1 requires SNF1 kinase activity.

Increased expression of SIP1 does not appear to suppress *snf4* defects by stimulating SNF1 kinase activity. Multiple copies of *SIP1* do not restore SNF1 kinase activity in an *snf4Δ* mutant but do allow weak detection of phosphorylated SIP1 protein (Fig. 4B). Taken together, our results suggest that SIP1 functions downstream of SNF1. One possibility is that SIP1 mediates the effects of the SNF1 kinase in the signaling pathway. Alternatively, SIP1 could facilitate interaction of SNF1 with other substrates.

These genetic and biochemical studies show that the interaction between SIP1 and the SNF1 protein kinase detected by the two-hybrid method reflects a functional interaction in vivo. The method is applicable to cloned kinase genes from any organism and is very sensitive; interactions yielding only a few units of β-gal activity can be detected in the color assay. Thus, the two-hybrid system should prove generally useful for identifying substrates and other proteins that interact with protein kinases.

REFERENCES AND NOTES

1. S. Fields and O.-K. Song, *Nature* **340**, 245 (1989).
2. C.-t. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991).
3. J. L. Celenza and M. Carlson, *Science* **233**, 1175 (1986).
4. S. Thompson-Jaeger, J. Francois, J. P Gaughran, K. Tatchell, *Genetics* **129**, 697 (1991).
5. A. Alderson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8602 (1991).
6. J. L. Celenza and M. Carlson, *Mol. Cell. Biol.* **9**, 5034 (1989).
7. J. L. Celenza, F. J. Eng, M. Carlson, *ibid.*, p. 5045.
8. Three pGAD libraries (*2*) of plasmids carrying fusions between GAL4(768-881) and yeast *Sau3*AI fragments, in each reading frame, were used to transform (*11*) yeast strain GGY::171 [G. Gill and M. Ptashne, *Cell* **51**, 121 (1987)], which is deleted for *GAL4* and contains *GAL1-lacZ*, carrying the GAL4(1-147)-SNF1 hybrid plasmid (*1*). Transformants on plates selective for the plasmids and containing glucose were replicated onto nitrocellulose filters and were incubated with the chromogenic substrate X-Gal [J. Breeden and K. Nasmyth, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643 (1985)]. Out of 144 blue colonies, 82 turned blue on reassaying after purification on selective medium. Of these, 27 colonies containing *GAL4* plasmids were identified by the polymerase chain reaction with primers complementary to nucleotides 1206 to 1229 and 2552 to 2528 of the *GAL4* sequence and template prepared by boiling yeast colonies. For the remaining 55 colonies, cultures were grown in medium lacking Leu, DNA was prepared, and the *LEU2*-marked library plasmid was recovered by transformation of the *leuB*$^-$ *Escherichia coli* strain BA1 to ampicillin resistance and Leu independence. Each plasmid was used to transform GGY::171 with and without GAL4(1-147)-SNF1. Sixteen plasmids caused blue colony color only in the presence of GAL4(1-147)-SNF1, ten plasmids conferred blue color independent of GAL4(1-147)-SNF1, and in the remaining cases the recovered plasmid did not cause a blue phenotype. We compared the 16 plasmids by colony hybridization of the bacterial clones (*14*). The plasmids pG_{AD}-$SIP1_{201}$ and pG_{AD}-$SIP1_{432}$ (named according to the codon at the fusion point) were from different libraries.
9. M. Carlson and D. Botstein, *Cell* **28**, 145 (1982).
10. E. J. A. Hubbard, X. Yang, M. Carlson, *Genetics* **130**, 71 (1992); L. C. Robinson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 28 (1992).
11. Standard yeast genetic methods were used throughout [M. D. Rose, F. Winston, P. Hieter, *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990)].
12. J. Ma and M. Ptashne, *Cell* **51**, 113 (1987).
13. L. Guarente, *Methods Enzymol.* **101**, 181 (1983); J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972).
14. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989).
15. D. M. Ruden, J. Ma, Y. Li, K. Wood, M. Ptashne, *Nature* **350**, 250 (1991).
16. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).

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Participation in Normal Immune Responses of a Metastasis-Inducing Splice Variant of CD44

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A variant of the glycoprotein CD44 (CD44v) that shares sequences with variants causally involved in metastasis formation is transiently expressed on B and T lymphocytes and macrophages after antigenic stimulation and in the postnatal period. Antibodies to the variant hinder in vivo activation of both B and T cells. The observation that a protein domain that is expressed on CD44 and required for the lymphatic spread of tumor cells can catalyze an essential step in the process of lymphocyte activation supports the idea that metastasizing tumor cells mimic lymphocyte behavior.

Tumor cells that metastasize by way of the lymphatics leave the site of local tumor growth and enter peripheral lymphatic organs through the afferent lymph. This process resembles the trafficking of lymphocytes. When lymphocytes contact antigen, they leave the periphery and enter the draining lymphatic tissue where they are selectively retained, activated, and expanded before reentry into the circulation (*1*).

We have recently observed that highly

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