matography on a nickel column according to the manufacturers protocols. Full-length a1 protein with six histidine residues fused to the COOH-terminus was expressed from plasmid pYJ173, a derivative of pET21a(+), and purified according to the protocol (Novagen). Electrophoretic mobility shift assays were performed as described (6). Reactions were incubated for 1 hour at RT and then electrophoresed on a nondenaturing 6% polyacrylamide gel with 0.5× TBE. Dried gels were exposed to a phosphor screen and the image was scanned on a Molecular Dynamics model 425 phosphorimager.
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Titins: Giant Proteins in Charge of Muscle Ultrastructure and Elasticity

Siegfried Labeit and Bernhard Kolmerer

In addition to thick and thin filaments, vertebrate striated muscle contains a third filament system formed by the giant protein titin. Single titin molecules extend from Z discs to M lines and are longer than 1 micrometer. The titin filament contributes to muscle assembly and resting tension, but more details are not known because of the large size of the protein. The complete complementary DNA sequence of human cardiac titin was determined. The 82-kilobase complementary DNA predicts a 3-megadalton protein composed of 244 copies of immunoglobulin and fibronectin type III (FN3) domains. The architecture of sequences in the A band region of titin suggests why thick filament structure is conserved among vertebrates. In the I band region, comparison of titin sequences from muscles of different passive tension identifies two elements that correlate with tissue stiffness. This suggests that titin may act as two springs in series. The differential expression of the springs provides a molecular explanation for the diversity of sarcomere length and resting tension in vertebrate striated muscles.

During the past decade, additional filament systems formed by two giant proteins, titin and nebulin, had to be incorporated into the established view of vertebrate striated muscle as a two-filament sliding system [for reviews see (*1*)]. Progress on the molecular characterization of the titin and nebulin filaments initially was limited to histochemical approaches and electron microscopy because of the unusual sizes of these molecules, with molecular weights in the megadalton range. Native molecules of titin (*2*), also referred to as connectin (*3*), are filaments >1 μm long (*4*) that in situ extend from Z discs to M lines (*5*) (Fig. 1). The portion of titin that spans the A band (the region within the thick filament, referred to here as A band titin) is composed of regular arrangements of domains (*5*) that bind to other proteins of the thick filament (*6*, *7*). This suggests that titin is involved in the regulation of the A band ultrastructure. In the I band (the region of titin that spans the thin filament), titin filaments are extensible, as revealed by immunoelectron microscopy (*5*, *8*). This is likely to account for the intrinsic elasticity of vertebrate striated muscle myofibrils (*9*), because degradation of titin by radiation or proteases or its removal by extraction results in a loss of passive tension (*10*). The critical role of the titin filament for muscle structure and function cannot be unraveled without knowledge of its primary structure. Here, we determined the complete complementary DNA (cDNA) sequence of human titin. We suggest that titin specifies a sarcomeric building plan, into which tissue-specific features such as different passive tensions are provided by differential splicing.

We have previously isolated five partial titin cDNA clones from a human heart cDNA library (*6*). The partial titin cDNAs have been extended systematically into both the 5′ and the 3′ direction by anchored polymerase chain reaction (PCR) techniques. A total of 49 extensions were found to link the partial cDNAs into one 82-kb sequence contig. This contig represents the full-length coding sequence of titin expressed in the human heart, as indicated by the presence of untranslated regions at the 5′ and 3′ ends. The 81-kb open reading frame predicts a 26,926-residue protein with a molecular mass of 2993 kD. Ninety percent of the mass is contained in a repetitive structure composed of 244 copies of 100-residue repeats (Fig. 2). These repeats encode 112 immunoglobulin (Ig)-like and 132 FN3-like domains. The pool of 54 partial cardiac titin cDNAs identified 60 λ phage clones in a human genomic DNA library to which the partial cDNAs were assigned in a co-linear order. A subset of 16 phage clones was sufficient to span the 54 cardiac titin cDNAs and covered a contiguous span of 300 kb of genomic DNA. This argues against the presence of cloning artifacts and also suggests that the portion of the titin gene harboring the coding se-

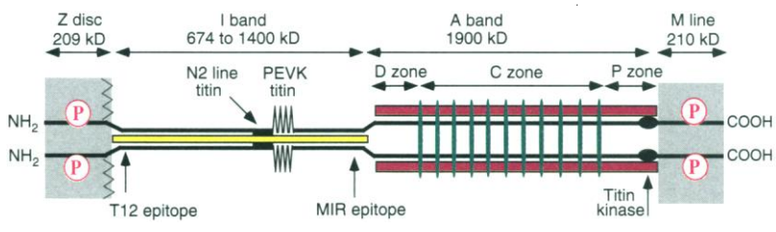

Fig. 1. Model for titin in the sarcomere. The titin filament is shown in black, the thin filament (actin) in yellow, and the thick filament (myosin) in red. The epitopes of the titin antibodies T12 and antibodies to the MIR have been mapped in the sarcomere by immunoelectron microscopy (*5*, *26*); the positions of their epitopes in the titin sequence are known (*27*). Antibodies to the titin kinase domain react with the periphery of the M line (*12*). Therefore, it can be estimated which sections of the titin sequence are in the Z disc, I band, A band, and the M line. For the I band, the range of variation as predicted by the observed splice variants is indicated. The presumed extensible element of the I band, the PEVK element, is located between the N2 line titin and the second tandem Ig block (zig-zag pattern). Within the thick filament in the central C zone (green stripes), titin binds to both the C protein and myosin (*7*, *8*) and is likely to specify the presence of 11 copies of the 430 Å thick filament repeat in vertebrate striated muscles. Phosphorylation of tandemly arranged Ser-Pro repeats in the Z disc and the M line titin (red P) may control integration of the titin filament into Z discs and M lines during myogenesis (*13*).

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quences is ~300 kb in size.

The epitope position of the T12 titin antibody predicts that about 200 kD of titin enters the Z disc (Figs. 1 and 2). Domain architecture in the Z disc region of titin is arranged by a specialized class of Ig-like domains alternating with nonrepetitive sequences. The I band region of titin is composed of three different segments: tandem Ig-like domains, nonrepetitive sequence insertions in the central region of the I band, and a complex domain architecture near the junction of the A band and the I band. This correlates with earlier electron microscopic data that show that the I band titin has a complex fine structure (*11*). The nonrepetitive sequences in the central I band region (Fig. 2) are possibly involved in the self-association of the two or three titin strands (or half sarcomere) seen in the N2 line region. The end of the tandem Ig segment and the transition into a more complex architecture toward the A band (Fig. 2) may correspond to the junction point where titin strands branch from the thin filament to enter the thick filament (*11*).

For the A band section, where detailed ultrastructural information is available, the correlation of titin sequence and sarcomere structure is most obvious. Seven-module super-repeats correspond to the D zone titin, 11-module super-repeats to the C zone titin, and a less regular P zone region connects the A band to the M line titin (Fig. 2). The M line titin encompasses about 200 kD at the COOH-terminal end of titin (*12*). Its structure has been described in detail (*13*).

Some monoclonal antibodies specific to the I band titin distinguish between the cardiac and skeletal titins (*14*), and electrophoretic mobilities indicate that titins from different tissues have different sizes (*15*). Therefore, we compared human and rabbit titin mRNAs from different tissues. In the central part of the I band, cardiac and skeletal titins branch into distinct isoforms (Fig. 3). The branch points correspond to the exon-intron boundaries of the genomic sequence, which indicates that alternative splicing accounts for the tissue-specific variation of the I band titin structure (*16*). In heart, differential splicing includes about 3.5 kb of cDNA between domains I15 and I20 (where I15 and I20 are the 15th and 20th 100-residue segments, respectively, within the I band region of titin); in skeletal muscle, 22.5 kb of cDNA is included (Fig. 3). The inclusion of two structural motifs in different copy numbers accounts for the tissue-specific variation of I band titin mass (*17*). First, cardiac titin comprises 37 tandem Ig repeats, whereas 90 tandem Ig domains are present in the human skeletal sequence. Second, a sequence element rich in Pro (P), Glu (E), Lys (K), and Val (V) residues has a different length in cardiac and skeletal muscle (Fig. 4). The four amino acids P, E, V, and K constitute 70% of this element, and therefore we refer to it as the PEVK domain of titin. This domain comprises 163 or more residues in the cardiac titin sequence and 2174 residues in the skeletal titin sequence.

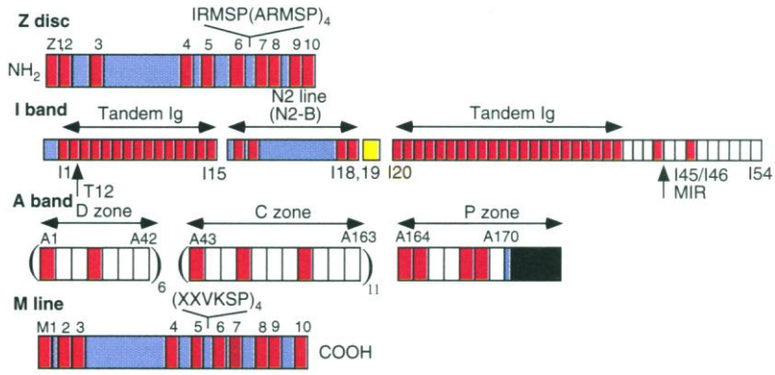


Fig. 2. Domain structure of the cardiac titin filament. The modular architecture of cardiac titin as predicted by its full-length cDNA is shown. A total of 244 copies of 100-residue repeats (indicated by vertical rectangles) are contained, of which 112 belong to the Ig (red) domain and 132 to the FN3 (white) superfamily. The 100-residue repeats are indicated by region and position regardless of whether they are Ig or FN3 domains. The titin kinase domain is shown in black, the PEVK element (N2-B 163-residue variant; see Fig. 3) in yellow. Sequences with no homology to database entries comprise 10% of the titin primary structure (blue). The epitope positions of T12 and MIR are indicated (*28*). The change in motif organization NH_2-terminal of T12 is proposed to be the Z disc–I band junction; the start of super-repeats COOH-terminal of MIR is proposed to be the beginning of the A band region of titin. Within the A band region, the D zone contains six copies of the seven-module super-repeat (A1 through A42); the C zone contains 11 copies of the 11-module super-repeat (A43 through A163). The positions of the tandemly repeated RMSP and VKSP motifs in the Z disc and M line region of titin are shown (*29*).

Hybridization of the 22.5 kb of skeletal cDNAs included between the I15 and I20 branch sites to blotted RNAs from different rabbit striated muscles revealed a tissue dependence of signal intensities with probes from the tandem Ig and PEVK region (Fig. 5). Different isoforms of I band titin were also detected within the same tissue. In heart, many splice variants occur within the PEVK region (Fig. 4), and two distinct isoforms of N2 line titin, N2-A and N2-B, were observed (Fig. 3). We conclude that a range of isoforms of I band titin is generated by differential splicing, which affects the length of the tandem Ig and the PEVK region of titin.

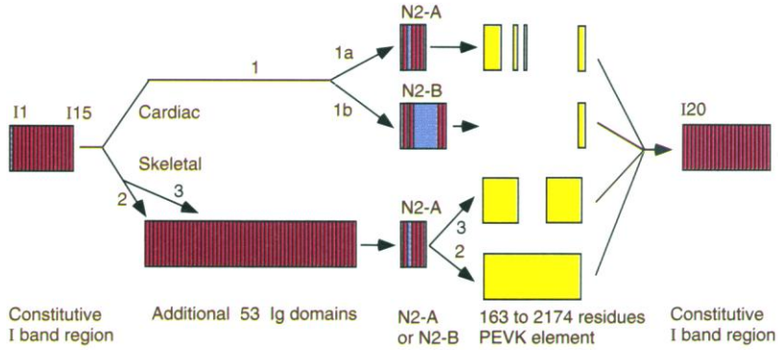


Fig. 3. Differential splicing pathways of I band titin. Comparison of human cardiac and skeletal titin cDNAs (*30*) reveals branching into distinct sequences between I15 and I20; color codes for domains are as in Fig. 2. In splicing pathway 1, for the heart, alternative splicing includes one of two distinct elements, termed N2-A and N2-B; the homology of N2-A to a partial chicken titin sequence from the N2 line region (*31*) suggests that N2-A and N2-B encode N2 line titin. Two sequenced variants of the PEVK element that are linked to the cardiac pathway N2-B encompass 163 and 181 residues (1b). N2-A–associated PEVK elements appear to be larger (1a). In splicing pathway 2, for skeletal muscle, 53 additional copies of tandem Ig domains are included, and the PEVK element is 2174 residues long. RT-PCR analysis of human tissues (*32*) with primer pairs derived from sequences from splicing pathways 1 and 2 suggests the presence of splicing pathway 2 in human soleus and diaphragm tissue. In human psoas tissue, some of the tandem Ig and PEVK sequences are excluded, and the approximate regions of excluded sequences are given (3). The human cardiac sequence submitted to the EMBL data library corresponds to pathway 1b, the skeletal I band sequence to pathway 2.

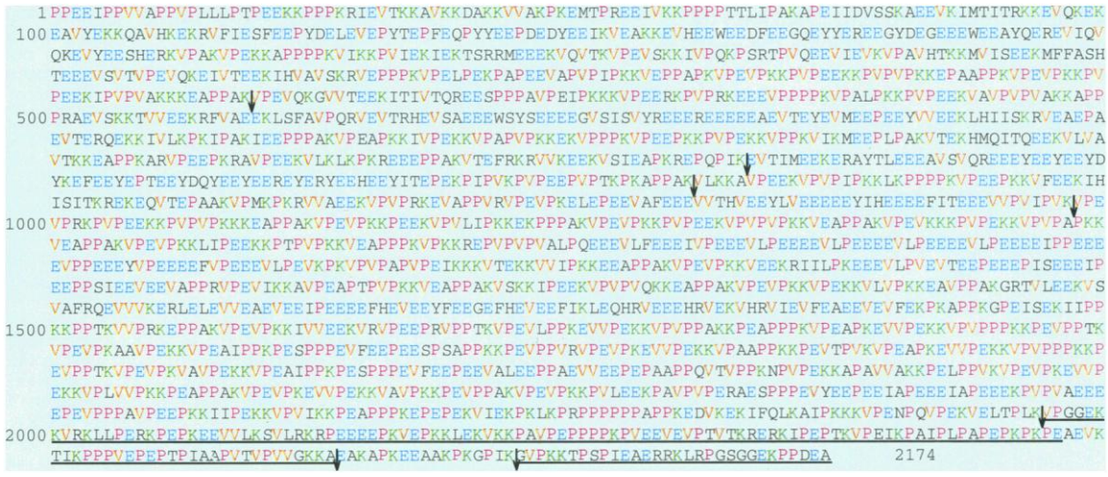


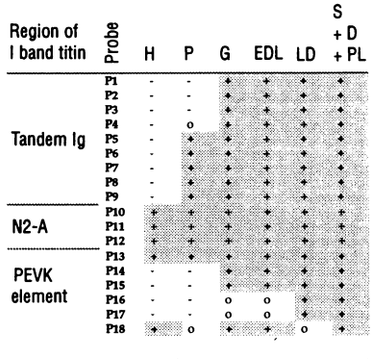
Fig. 4. The PEVK region in titin. The 2174 residues of the PEVK element from the human skeletal entry are shown (*29*). It is composed predominantly of four residues (P, pink; E, blue; K, green; V, yellow), and Pro-rich regions alternate with stretches rich in Glu residues. Analysis of cardiac cDNA clones from the PEVK region reveals multiple positions of differential splicing within the PEVK sequences (indicated by arrows), which suggests that the PEVK element can be cut in many different ways. The underlined 163 residues are shared by all sequenced cardiac clones and correspond to the N2-B–associated splice pathway (Fig. 3).

Vertebrate thick filaments are organized into D, C, and P zones (*18*). It has not been understood what regulates thick filament assembly into three structurally distinct compartments and why 11 stripes of members of the MyBpC protein family (C, H, and X protein) are bound at regular intervals of 430 Å in the C zone (*18*). The C zone titin sequence comprises 11 copies of 11-module super-repeats, which bind to the C protein in vitro (*6*, *7*). We conclude that the super-repeat architecture of A band titin can explain the global architectures present in vertebrate thick filaments. The high degree of sequence conservation in the C zone titin (*6*, *19*) correlates with the presence of C zones in all vertebrate thick filaments. In contrast to the conserved module architecture of the A band titin, the structure of I band titin depends on tissue type (Fig. 3). Also, in the M line, two distinct isoforms of titin are expressed in correlation with the M line fine structure (*20*). Therefore, we suggest that where sarcomeres have a conserved ultrastructure, the domain architecture is constant, whereas tissue-specific features are introduced into the building plan of the titin mRNA by differential splicing in regions where sarcomere ultrastructures are variable.

Comparison of the I band titin sequences from heart, psoas, and soleus muscles, each of which has different passive tensions and expresses different size classes of titin (*15*), identifies the regions that are involved in elasticity. The I band sequences have different contents both of the tandem Ig and of the PEVK segments. Previous work on the molecular basis of elasticity in titin assumed that I band titin is composed only of Ig and FN3 domains. Therefore, it was proposed that the Ig-like β barrel structures of the titin repeats (*21*) unfold to gain an increase in length of severalfold (*22*). However, the stability of expressed I band domains does not support such unfolding theories (*23*). The PEVK region might be more easily stretched than the Ig domains because the reduced complexity of the sequence together with the clusters of negative charges should prevent the formation of stable tertiary structure folds. In an extended state, the 2200-residue version of the PEVK segment could span up to 0.8 μm in vivo and account for the initial ~0.8-μm extension per half sarcomere, during which most skeletal muscles develop only little passive tension (*15*).

Therefore, the PEVK domain may account for the extensibility of the titin filament at low forces. After this extensibility has been exhausted, the stable folds of the titin's I band Ig domains will resist further extension. This would explain the rapid rise in tension toward the end of the physiological slack length of sarcomeres. The moderate levels of further extension (beyond 0.8 μm per half sarcomere) that occur in vivo in skeletal muscles may be explained by conformational changes in the tandem Ig segments, such as bending and stretching of interdomain linkers. In conclusion, we propose that the PEVK region and tandem Ig titin are two elements of differential stiffness that function in parallel as a two-spring system. This is in agreement with mechanical studies on muscle that propose a two-spring model (*15*, *24*), and with immunohistochemical studies showing a nonuniform extensibility within the I band titin (*11*, *25*).

It remains to be seen how the multiple splicing events in the I band titin are regulated. The length of the tandem Ig and the PEVK segments are correlated, presumably because the longer slack lengths of more elastic muscles necessitate longer I bands. Therefore, their splicing must be coordinated. The second giant muscle protein nebulin is thought to specify the length of the thin filament and is also expressed in variable length versions by differential splicing (*26*). The assembly plans of the I band that are specified by both molecular rulers will match only if differential splicing events controlling the size of I band titin and



Region of I band titin	Probe	H	P	G	EDL	LD	S + D + PL
Tandem Ig	P1	-	-	+	+	+	+
	P2	-	-	+	+	+	+
	P3	-	-	+	+	+	+
	P4	-	o	+	+	+	+
	P5	-	+	+	+	+	+
	P6	-	+	+	+	+	+
	P7	-	+	+	+	+	+
	P8	-	+	+	+	+	+
	P9	-	+	+	+	+	+
N2-A	P10	+	+	+	+	+	+
	P11	+	+	+	+	+	+
	P12	+	+	+	+	+	+
	P13	+	+	+	+	+	+
PEVK element	P14	-	-	+	+	+	+
	P15	-	-	+	+	+	+
	P16	-	-	o	o	+	+
	P17	-	-	o	o	+	+
	P18	+	o	+	+	o	+

Fig. 5. The type of I band titin expressed depends on the tissue. Expression of the 22.5-kb of central I band titin sequences located between I15 and I20 was monitored in a panel of striated muscle RNAs (*33*). Total RNA from the indicated muscles was blotted onto nylon membranes and hybridized to a set of probes (P1 through P18) covering the 22.5-kb region. Analyzed tissues were all from rabbit. H, heart; P, psoas; G, gastrocnemius; EDL, extensor digitorum longus; LD, longissimus dorsi; S, soleus; D, diaphragm; PL, plantaris longus. Signals were compared with a titin kinase probe, because the exon encoding this domain is expressed in all striated muscles (*20*). Expression less than 25% of the kinase control probe is indicated with a minus; for 60 to 100% of controls, with a plus; for values 25 to 60% of the control, as "o" (unspliced intermediates or splicing within the probe sequence may explain intermediate signals). The differential hybridization of tandem Ig and PEVK probes suggests that they have different lengths in different muscles.

nebulin are interdependent. Therefore, the precision and tissue specificity of the sarcomeric assembly program in vertebrates appear to necessitate coordinated splicing decisions in the titin and nebulin precursor mRNAs.

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5. In the microscope, striated muscle appears as a periodical array of darker and brighter stripes. The dark segment contains the thick filament (myosin) and is referred to as the A band, the bright segment contains the thin filament (actin) and is called the I band. In the center of A and the I bands, dense structures ~100 nm wide are present, referred to as M lines and Z discs, respectively. A single titin molecule spans the distance from the Z disc to the M line [D. O. Fürst, M. Osborn, R. Nave, K. Weber, *J. Cell Biol.* **106**, 1563 (1988)].
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28. For the main immunogenic region (MIR) of titin, patients' sera (*27*) were used to screen a human cardiac gt11 library, and the isolated phage inserts were expressed in *Escherichia coli*. Protein immunoblots and enzyme-linked immunosorbent assays with recombinant titin antigens assigned the MIR to I45 and I46 [M. Gautel *et al.*, *Neurology* **43**, 1581 (1993)]. T12 (*5*) monoclonal antibody was used to screen a gt11 library. Positive phages were sequenced from both ends and identified a region of overlap within I2 and I3.
29. Single-letter abreviations for the amino acids 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; Y, Tyr; and X, any residue.
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33. The 22.5 kb of skeletal I band titin included between I15 and I20 were amplified by PCR as a set of 18 subfragments. Total RNA was blotted on nylon membranes, cross-linked, and hybridized as described, except that temperatures were lowered to 55°C to account for the species difference of the probe and RNA [G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. Probes P1 through P18 were labeled randomly essentially as described [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **13**, 6 (1983)].
34. We thank J. Trinick for his contributions in the initial stages of this project, M. Saraste, A. Pastore, B. Bullard, K. Leonard, and M. Gautel for their continuous support and encouragement, D. O. Fürst and K. Weber and C. Witt and H. Jockusch for communicating unpublished results, and the Chirugische Kinik Heidelberg for human muscle tissues from organ transplant donors. We gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft, the European Community, the Human Frontier Science programme, and the Frauenklinik Mannheim. The 82-kb cardiac titin cDNA and the 22-kb I band titin cDNA included in soleus skeletal muscle have been submitted to the European Molecular Biology Laboratory (EMBL) data library (accession numbers AC X90568 and X90569, respectively). The coordinates of the P1 through P18 subfragments are included in the annotations to accession number X90569; the DNA is available from us. Additional information on the titin sequence and available cDNA and genomic probes can be obtained from the World Wide Web from http://www.aaas.org/science/science.html; see "Beyond the Printed Page."

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Requirement for Generation of H_2O_2 for Platelet-Derived Growth Factor Signal Transduction

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Stimulation of rat vascular smooth muscle cells (VSMCs) by platelet-derived growth factor (PDGF) transiently increased the intracellular concentration of hydrogen peroxide (H_2O_2). This increase could be blunted by increasing the intracellular concentration of the scavenging enzyme catalase or by the chemical antioxidant *N*-acetylcysteine. The response of VSMCs to PDGF, which includes tyrosine phosphorylation, mitogen-activated protein kinase stimulation, DNA synthesis, and chemotaxis, was inhibited when the growth factor–stimulated rise in H_2O_2 concentration was blocked. These results suggest that H_2O_2 may act as a signal-transducing molecule, and they suggest a potential mechanism for the cardioprotective effects of antioxidants.

Evidence from both plant and animal cells suggests that H_2O_2 may act as an intracellular second messenger. Hydrogen peroxide may regulate the defense of plants against viral pathogens by serving as a small diffusible molecule to orchestrate the hypersensitive response (*1*). Salicylic acid binds to and inactivates tobacco catalase, leading to a rise in H_2O_2 concentration ($[H_2O_2]$) and the activation of gene expression (*2*). In mammalian cells, H_2O_2 has been implicated as an indirect activator of the transcription factor nuclear factor kappa B (NF-κB) (*3*). Because NF-κB modulates the expression of a variety of immune and inflammatory molecules, it would appear that a role for H_2O_2 in host defense mechanisms has been conserved from plants to animals. Similarly, H_2O_2 may function in triggering apoptosis in both plant and animal cells (*1*, *4*).

Stimulation of various mammalian cell types with either cytokines, phorbol esters, or growth factors increases the secretion of

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