cumulative changes occur in TAg-expressing mice between 4 and 7 months of age that prohibit reversal of cellular transformation even in the absence of the primary transforming stimulus. The nature of the these changes remains to be identified.

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20. Salivary gland tissue was homogenized in 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, phenylmethylsulfonyl fluoride (10 mg/ml), aprotinin (30 μl/ml) (Sigma), and 100 mM sodium orthovanadate in phosphate buffered saline. Proteins (20 μg) were separated in an 8% SDS–polyacrylamide gel and transferred onto a NOVEX polyvinylidene difluoride membrane by means of a NOVEX Western Transfer Apparatus. After transfer and blocking with buffer [5% nonfat milk, 10 mM tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20] for 1 hour at room temperature, the membrane was exposed to a 1:400 dilution of mouse monoclonal antibody Pab 101 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by exposure to a 1:4000 dilution of peroxidase-conjugated rabbit polyclonal antibody to mouse immunoglobulin G (Jackson Immunochemical, West Grove, PA) for 1 hour at room temperature. The proteins were visualized with the ECL protein immunoblotting protocol (Amersham).
21. Slow-release tetracycline pellets (Innovative Research of America, Toledo, OH) were implanted subcutaneously in the shoulder region with the use of a trochar according to the manufacturer's directions. These pellets released 0.7 mg of tetracycline hydrochloride per day over a 21-day period.
22. P. A. Furth, M. Li, L. Hennighausen, unpublished data.
23. P. A. Furth and L. Hennighausen, unpublished data. Quantative analyses on two mice revealed that approximately 40% of the ducts were hyperplastic.
24. The degree of ploidy was determined on Feulgen-stained paraffin sections.
25. L. Hennighausen and G. Auer, unpublished data.

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Correction of the Mutation Responsible for Sickle Cell Anemia by an RNA-DNA Oligonucleotide

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A chimeric oligonucleotide composed of DNA and modified RNA residues was used to direct correction of the mutation in the hemoglobin β^S allele. After introduction of the chimeric molecule into lymphoblastoid cells homozygous for the β^S mutation, there was a detectable level of gene conversion of the mutant allele to the normal sequence. The efficient and specific conversion directed by chimeric molecules may hold promise as a therapeutic method for the treatment of genetic diseases.

Sickle cell anemia is the classic prototype of a hereditary hemoglobinopathy resulting from a point mutation in the β-globin gene (*1*, *2*). The clinical condition results from homozygosity of the sickle cell allele β^S, in which an A-to-T mutation within the sixth codon of the β-globin coding region changes the normal glutamic acid residue to a valine. The severity and prevalence of this disease have made sickle cell anemia a candidate for gene therapy. Over the past several years a variety of strategies for genetic modification have been devised. These have included transduction methods based on retrovirus and adeno-associated virus vectors to deliver the normal β- or γ-globin gene to hematopoietic progenitor cells (*3*). Nevertheless, the attendant random integration of retrovirus vectors and genomic rearrangements accompanying integration of adeno-associated viruses are undesirable consequences of gene transfer mediated by viral vectors. Correction of the β^S allele to β^A through a gene conversion mechanism would provide a means for gene therapy that circumvents this problem.

In a recent study from this laboratory, a procedure was reported for correcting the mutated form of an extrachromosomal gene present on a plasmid by use of a chimeric oligonucleotide composed of DNA and RNA residues (*4*). The design of this molecule was prompted by the discovery that RNA-DNA hybrids were highly active in homologous pairing reactions in vitro and that hairpin caps at the ends of hybrid molecules were no impediment to pairing (*5*). These observations suggested a strategy for targeted correction in which a short, double-stranded oligonucleotide vector is activated for recombination by incorporating RNA residues and protected from exonucleolytic degradation by capping both ends. The 2′-O-methyl modification of ribose of the RNA added protection against cleavage by ribonuclease (RNase) H activities.

To correct the β^S mutation, we designed the chimeric oligonucleotide (SC1) as a single molecule (with two sequences that were inverted and complementary) capable of folding back on itself to form a duplex structure (Fig. 1). The molecule was composed of DNA residues with two intervening blocks of 10 2′-O-methyl RNA residues flanking a short stretch of five DNA residues. When the molecule was folded into the duplex conformation, the sequence of one strand comprised all DNA residues whereas the other strand contained the RNA-DNA blocks. In this case, the internal sequence is complementary to the β^S-globin sequence over a stretch of 25 residues that span the site of the β^S mutation, with the exception of a single base (T). The five DNA residues flanked by RNA residues were centered about the mutant T residue in the β^S coding sequence. A control chimeric oligonucleotide (SC2) was designed in the same manner with the exception of one base (A; designated in bold and with an asterisk in Fig. 1).

The chimeric molecule was introduced into lymphoblastoid cells (B cells) homozygous for the β^S allele by means of a commercial liposome formulation, and the cells were assayed for correction after 6 hours (*6*, *7*). Correction of the single base mutation was assessed by taking advantage of restriction fragment length polymorphisms (RFLPs) resulting from the β^S mutation (*2*): the A-to-T transversion in the β^S allele results in the loss of a Bsu 36I restriction

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site (CCTGAGG). A 1.2-kbp Bsu 36I DNA fragment of the β-globin gene is absent in the β^S allele and is replaced by a diagnostic 1.4-kbp fragment (8). When genomic DNA recovered from homozygous β^S lymphoblastoid cells was analyzed with this procedure, the 1.4-kbp fragment was observed (Fig. 2). However, two fragments were observed in DNA from cells transfected with the chimeric molecule. The presence of the 1.2-kbp fragment in addition to the 1.4-kbp fragment indicates that the β^S allele was partially corrected in a dose-dependent fashion.

To measure the efficiency of correction rapidly and sensitively, we adapted a polymerase chain reaction (PCR)–based RFLP analysis (9). Primers were designed to yield a 345-bp fragment spanning the site of the β^S mutation after PCR amplification of genomic DNA. The fragment from normal cells contains a Bsu 36I recognition sequence and yields fragments of 228 and 117 bp upon digestion, whereas β^S DNA contains the sequence CCTGTGG and remains refractory to cutting. Analysis indicated that the 345-bp DNA fragment amplified from SC1-treated β^S cells was partially cleaved with Bsu 36I, indicating correction of the mutation on some but not all chromosomes (Fig. 3). The frequency of correction was dependent on the dose of the chimeric molecule (SC1) used in transfection.

Direct sequencing of the PCR-amplified 345-bp fragment was performed to confirm the T-to-A change in the coding strand. In the DNA sample from β^S cells transfected with chimeric molecule SC1 (at a higher concentration than used in the RFLP mapping), sequence analysis revealed an approximately equal mixture of A and T residues at the site of the β^S mutation (Fig. 4A). DNA from untreated β^S cells contained only T at that position and DNA from β^A cells contained only A whether treated with SC1 or not (Fig. 4A) (*10*). β^S cells transfected with the control chimeric molecule SC2 remained unchanged in sequence after treatment (Fig. 4A). However, some of the DNA from normal cells transfected with SC2 was partially converted to the β^S mutant sequence as evidenced by a mixture of T and A residues at the site altered in the β^A allele (Fig. 4A). These results indicate that the chimeric molecule can direct introduction or correction of mutations in the genome.

Because the β-globin gene is a member of a family of related genes, there was the possibility that one or more of the other globin genes might have been inadvertently altered as a consequence of transfection with the chimeric molecule. The most likely candidate for alteration was the δ-globin gene because it is more than 90% homologous to the β-globin gene. There is complete identity over the 5-bp DNA-core targeting region of the chimeric molecule SC1 and two single-base differences characterizing regions corresponding to the flanking RNA stretches. To investigate whether there were alterations introduced into the δ-globin gene after transfection of cells with the chimeric molecules, we sequenced δ-globin DNA as described above. When SC1 was used to transfect cells, no change was found in the δ-globin sequence over the region of DNA homology spanned by the chimeric molecule (Fig. 4B). There was likewise no change found in the δ-globin sequence of DNA when SC2 was used to transfect cells (Fig. 4B). The outcome in this latter case is in contrast to the observed change directed by SC2 in the β^A-globin sequence (Fig. 4A). It is possible that the gene conversion mechanism responsible for the change might be inactivated when there is nonidentity in the sequence spanned by the RNA region.

As a minimal screen to measure the reactivity of the chimeric oligonucleotide on the rest of the genome, mutagenesis of the hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene was monitored. This gene encodes an enzyme in the purine salvage pathway, and because it is X-linked, events at a single allele can be measured by induction of resistance to 6-thioguanine (*11*). In this experiment (day −4), β^A cells containing a

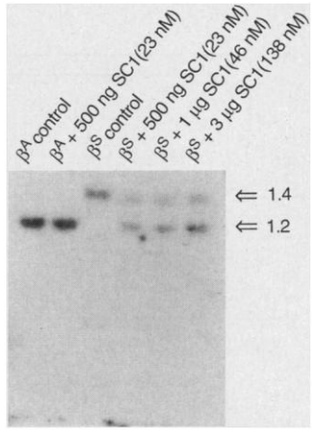


Fig. 2. Southern hybridization analysis of DNA from SC1-treated cells. Genomic DNA from β^A control cells and β^S cells transfected with SC1 was cut with Bsu 36I and analyzed by Southern hybridization. Globin DNA from β^A and β^S migrates as 1.2- and 1.4-kbp fragments, respectively, indicated to the right.

normal β-globin gene were transfected with SC2-DOTAP (Boehringer Mannheim) for 16 hours, and the conversion of the normal β-globin gene to the sickle cell genotype was analyzed with the PCR method described above (see Fig. 3). As shown in the inset of Fig. 4C, the cleavage of the PCR-generated fragment is reduced (β^A + SC2, day 0), indicating conversion from β^A to β^S. At this time (day 0), 6-thioguanine was added and cell survival was determined at various times up to 14 days. There was no significant difference in cell counts during this period between the cell population treated with the chimeric oligonucleotide and the untreated cells (Fig. 4C). On day 14, the conversion of β^A to β^S was measured in treated and untreated cells, and again, a detectable amount of PCR product remained uncleaved by Bsu 36I in the SC2-treated but not untreated samples, there-

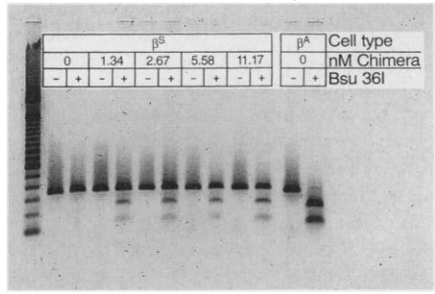
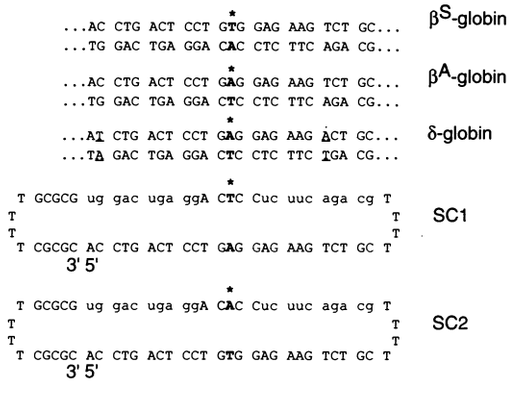


Fig. 3. SC1 directs correction of β^S to β^A in a dose-dependent manner. A 345-bp fragment spanning the site of the β^S locus was generated by PCR and digested with Bsu 36I. DNA from β^A contains the sequence CCTGAGG and is cut by Bsu 36I to yield 228- and 117-bp fragments whereas β^S DNA that has the sequence CCTGTGG is resistant to digestion.

Fig. 1. Sequences of the chimeric oligonucleotides and of β^S-, β^A-, and δ-globin. The site of mutation is indicated by the asterisk in the β^A and β^S sequences. The homologous sequence in the δ-globin gene is aligned with the corresponding sequence in the β-globin gene. The residues underlined in δ-globin differ from those in β^A. Chimeric molecule SC1 contains 25 bp of sequence identical to that in β^A, whereas the control oligonucleotide SC2 is identical to β^S. DNA residues are capitalized and 2′-*O*-methyl RNA residues are in lowercase.



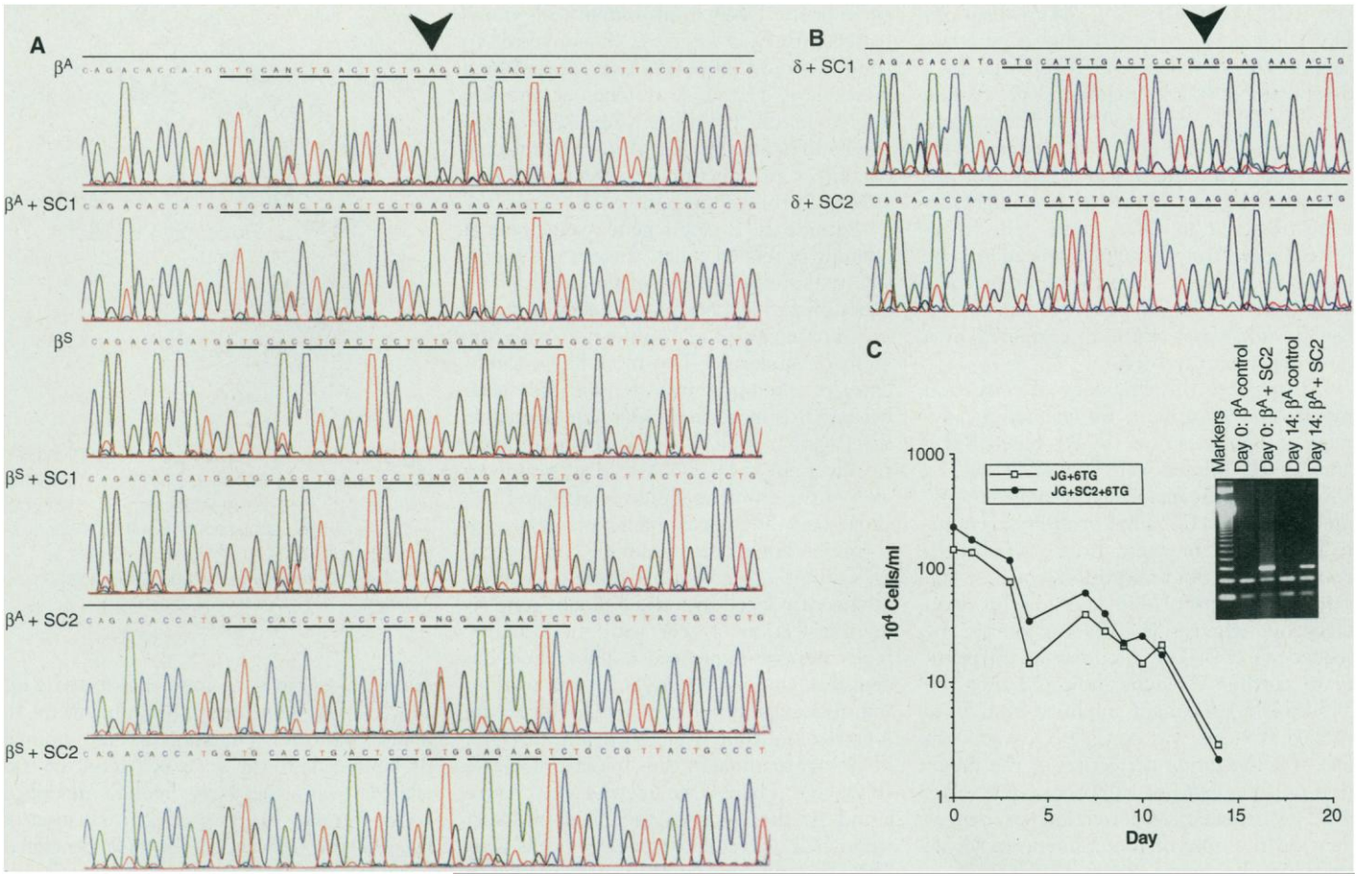


Fig. 4. (**A**) DNA sequence determination. Sequence analysis of the β- or δ-globin PCR products generated from cells transfected with 46 nM SC1 or SC2. The arrow designates the targeted base (A or T) for correction or conversion. (**B**) Sequence analysis of the δ-globin PCR products generated from cells transfected with 46 nM SC1 or SC2. The arrow designates the targeted base. (**C**) HPRT targeting as a measure of random mutagenesis induced by the chimeric molecule. Treated and untreated cells were incubated with 6-thioguanine (6TG) for the indicated times, and cell survival was measured directly by counting. PCR analyses were used to ensure that conversion occurred in treated cells at 0 and 14 days, respectively.

by confirming the results obtained on day 0. These observations suggest that treatment with a chimeric molecule in a lymphoblastoid cell induces the desired mutation conversion but is not generally mutagenic within the detection limits of the assay system. Hence, the possibility of random mutagenesis, although still present, is somewhat reduced.

This study shows that single base pair alterations in β-globin genes of cultured Epstein-Barr virus (EBV)–transformed cells can be introduced in the mammalian genome upon transfection with a chimeric RNA-DNA oligonucleotide. These findings could form the basis of an approach to gene therapy by providing an efficient method for site-directed changes in targeted genes. We are aware, however, that other genes may not respond to such treatment and thus extensive optimization studies for each target must be conducted.

Note added in proof: Conversion of the β-globin locus has not been successful in TK6 cells, even though the uptake of a radiolabeled chimeric oligonucleotide was slightly better than in the EBV-transformed B cells. The background mutagenesis level in the TK6 cells was 1.0×10^{-6}. The mutation frequencies in the SC1 and SC2 chimeric treated cells were 2.1×10^{-6} and 2.7×10^{-6}, respectively. There was partial conversion of the β-globin locus in K562 cells at 47 nM SC2, and these cells were also used for an HPRT mutagenesis experiment. The background mutagenesis frequency in these cells was $<1.3 \times 10^{-7}$. The number of 6TG-resistant colonies after treatment with SC2 was $<1.3 \times 10^{-7}$.

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6. Heparin-treated blood was obtained from discarded clinical material of a patient with sickle cell disease and from one of the investigators who had neither history nor symptoms of the disease. Mononuclear cells were prepared from blood (≈8 ml) with density-gradient centrifugation in Ficoll and infected with EBV that had been propagated in the marmoset cell line B95-8 (Coriell Institute for Medical Research catalog number GM07404D). Infections were performed with addition of 0.1 mg of lectin PHA-L (Sigma) in 10 ml of RPMI medium supplemented with 20% fetal bovine serum in a T25 flask. Cultures were fed twice a week starting on day 5 and were considered to be established once 60 to 70% of the cells remained viable at day 21.
7. The β^A and β^S lymphoid cells were maintained in RPMI medium containing 10% fetal bovine serum. Cells (1×10^5 per milliliter) were seeded in 1 ml of medium in each well of a 24-well tissue culture plate the day before the experiment. Transfections were performed by mixing chimeric oligonucleotides with 3 μg of DOTAP (Boehringer Mannheim) in 20 μl of 20 mM Hepes, pH 7.3; incubating the mixture at room temperature for 15 min; and adding the mixture to the cultured cells. After 6 hours the cells were harvested by centrifugation, washed, and prepared for PCR am-

plification (E. S. Kawasaki, in *PCR Protocols*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, New York, 1990), pp. 146–152.
8. For Southern blot analysis, 10 μg of genomic DNA was digested with Bsu 36I and the fragments separated by electrophoresis on a 0.7% agarose gel. After the DNA fragments were transferred to a nylon membrane, hybridization was performed with a ^{32}P-labeled, 1.2-kbp Bsu 36I β^A-globin fragment cloned in pBR328 as the probe.
9. For analysis of the β-globin sequence, the 345-bp PCR fragment was prepared by amplification from a crude cell lysate with primers BG02 (5′-TCCTAAGCCAGTGCCAGAAGA) and BG05 (5′-CTATTGGTCTCCTTAAACCTG) and Expand Taq polymerase (Boehringer Mannheim). For analysis of the δ-globin gene, the same cell extracts were used in amplification reactions with primers DG06 (5′-CTCACAAACTAATGAAACCCTGC) and DG07 (5′-GAAAACAGCCCAAGGGACAG) to generate a 335-bp fragment. Gels were stained with SYBR green (FMC Bioproducts), and fluorescence intensities were quantitated with a Molecular Dynamics fluorimager. DNA sequencing was performed in both directions with an ABI 373A sequencer.
10. There is a documented polymorphism in the third base of codon 2. A switch from C to T has been observed in this position and is evident in the β^A sequence [S. H. Orkin *et al.*, *Nature* **296**, 627 (1982)] outside the targeted region.
11. Four flasks were seeded with 24 ml of β^A cells at a concentration of 1×10^5 cells per milliliter. Two of the flasks were transfected for 16 hours with 47 nM SC2 chimeric oligonucleotide after which the medium was replaced. The cells rested for 3 days to allow for the expression of the HPRT-resistant phenotype. On day 0, 6-thioguanine was added (5 μg/ml) to each of the SC2-treated and untreated flasks. The media of these flasks was exchanged twice during the experiment. All cells were counted on day 0 and frequently thereafter for 14 days. The numbers are presented in Fig. 4C on a logarithmic scale, illustrating the kill curve of the 6-thioguanine–selected flasks. PCR analysis was performed on the control flasks on days 0 and 14 of the experiment, showing the persistence of the conversion event [H. L. Liber and W. G. Tilley, *Mutat. Res.* **94**, 467 (1982)].
12. We are indebted to members of E.B.K.'s laboratory for helpful suggestions throughout the course of this work, to H. Alder (Thomas Jefferson University) for assistance in the direct DNA sequencing protocols, and to A. Andrus (Applied Biosystems, Inc.) for providing the chimeric molecules. Supported by a grant from Kimeragen, Inc.

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Enhanced Protein C Activation and Inhibition of Fibrinogen Cleavage by a Thrombin Modulator

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A modulator of the enzymatic activity of human thrombin, designated LY254603, was identified that enhances the thrombin-catalyzed generation of the anticoagulant factor activated protein C, yet inhibits thrombin-dependent fibrinogen clotting. By means of mutant substrates, it was shown that LY254603 mediates the change in enzymatic substrate specificity through an alteration in thrombin's S3 substrate recognition site, a mechanism that appeared to be independent of allosteric changes induced by either sodium ions or by thrombomodulin. This compound may represent the prototype of a class of agents that specifically modulates the balance between thrombin's procoagulant and anticoagulant functions.

The serine protease thrombin is the pivotal controlling factor of all thrombosis, a leading cause of death as well as of morbidity associated with vascular disease. This enzyme plays a dual role in the regulation of blood coagulation. Thrombin's classical role is as a procoagulant enzyme that catalyzes the conversion of fibrinogen to fibrin, but thrombin also acts as an anticoagulant enzyme by the proteolytic activation of protein C, a key feedback regulator of the coagulation cascade (*1*). These opposing functions are physiologically controlled by allosteric changes induced by Na^+ (*2*) and cell-surface thrombomodulin (TM) (*3*), which results in a change in thrombin's substrate specificity. TM functions by directly blocking thrombin's procoagulant activities (*3*) and stimulating the thrombin-catalyzed generation of activated protein C (aPC) in the presence of Ca^{2+} (*4*, *5*).

Thrombomodulin's interaction with thrombin via anion-binding site I (*6*) results in an allosteric change that alters thrombin's substrate specificity (*7*). This allosteric change, at least in part, eliminates repulsive interactions between Glu^{39} and Glu^{192} in thrombin (*3*) and the P3 and P3′ acidic residues in protein C, which have been shown to contribute to the slow rate of activation by free α-thrombin (*5*, *8*). In addition to these allosteric changes in active-site substrate specificity induced by macromolecular interactions at anion-binding site 1, recent studies by Di Cera *et al.* (*2*) have demonstrated that thrombin exists in two forms allosterically controlled by Na^+ binding. The "fast" form of thrombin (Na^+-bound) cleaves fibrinogen with higher specificity, whereas the "slow" form preferentially cleaves protein C.

We reasoned that if thrombin existed in multiple forms capable of recognizing different substrates, then it might be possible to identify agents that would shift the equilibrium from one form to the other. Therefore, we tested approximately 8300 synthetic organic compounds from the Lilly Research Laboratories files (*9*) for agents that might increase the rate of thrombin-dependent activation of human protein C. Compounds that had a stimulating effect on protein C activation by thrombin were further analyzed for their effect on fibrinogen cleavage. Compounds with both thrombin activities were inspected for structural similarities, and, on the basis of that analysis, additional analogs were obtained from Lilly Research Laboratories files for comparative biochemical evaluation. After two iterations of this approach, LY254603 was identified (Fig. 1A, inset). At the screening concentration of 25 μg/ml, the rate of thrombin-catalyzed aPC generation was substantially increased in the presence of LY254603, as shown by an approximate eightfold increase in the amount of aPC generated (Fig. 1A). The compound had no effect on aPC amidolytic activity, as demonstrated in control experiments with preactivated protein C. Whereas the presence of LY254603 resulted in a marked stimulation in the ability of thrombin to cleave the protein C substrate, it also resulted in a marked reduction in the ability of thrombin to cleave fibrinogen (Fig. 1B). These data suggested that we identified a compound capable of altering the substrate specificity of thrombin.

The effects of LY254603 on stimulation of protein C cleavage and reduction of fibrinogen cleaving ability were dependent on concentration. The concentration required to double the rate of aPC generation in our assays was approximately 500 nM, and the maximal effect in this experiment reached a plateau at approximately a 10-fold increase (Fig. 1C). This plateau appeared to be a result of the solubility of the compound in the reaction, as the maximal water solubility was observed to be approximately 25 μM. The inhibitory effect of LY254603 on thrombin cleavage of fibrinogen was concentration-dependent within the same range, as observed for the stimulating effect on protein C cleavage (Fig. 1D). In repeated experiments, the median inhibitory concentration (IC_{50}) for fibrinogen clotting and the concentration for half-maximal stimulation of protein C cleavage were both ~3 μM. The ~10-fold increase in aPC generation with the more than 95% decrease in fibrinogen clotting (at the same concentrations of both thrombin and LY254603) effectively yields a shift of at least several hundredfold in thrombin's differential cleavage specificity. LY254603 significantly extended the activated partial thromboplastin clotting time in human plasma (16 ± 5 s at 40

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