13

DNA Topoisomerase Modification

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I. INTRODUCTION

The first reports of topoisomerase modification were published in 1982 and 1983 (Mills et al. 1982; Durban et al. 1983; Ferro et al. 1983; Jongstra-Bilen et al. 1983). Although a wide variety of posttranslational modifications of DNA topoisomerases may occur, this chapter focuses only on phosphorylation and poly(ADP-ribosylation), which have been observed both in vitro and in vivo. The experimental data suggest a regulatory role, but the precise cellular functions of these DNA topoisomerase modifications remain undefined at the present time.

A. Phosphorylation of DNA Topoisomerase at Serine Residues

The initial reports of phosphorylation in topoisomerases involved topoisomerase I purified from Novikoff hepatoma cells. A phosphoprotein of approximately 110 kD with a pI of 8.4 purified from cells labeled with ^{32}P (Durban et al. 1981) was subsequently identified as DNA topoisomerase I (Durban et al. 1983). A form of topoisomerase I could also be labeled in vitro by incubation with serine-specific protein kinases and [^{32}P]ATP. A protein kinase from an African Burkitt's lymphoma cell line that appeared to exhibit high affinity for topoisomerase I was purified (Mills et al. 1982). This serine kinase had an apparent K_m of ap-

DNA Topology and Its Biological Effects

proximately 0.3 μM for topoisomerase I; phosphorylation stimulated the DNA-relaxing activity of the enzyme three- to fivefold. The discovery of phosphorylation of topoisomerase I was followed by reports of phosphorylation of topoisomerase II. Sander et al. (1984) found a protein kinase activity that remained associated with *Drosophila* topoisomerase II through four purification steps. Phosphoserine was the predominant species modified by the topoisomerase-associated protein kinase.

Because phosphorylation and dephosphorylation of proteins are recognized as major mechanisms for regulating cellular functions, the reports of DNA topoisomerase phosphorylation stimulated studies with purified protein kinases. Casein kinase II, a messenger-independent kinase that uses acidic proteins like casein as substrate (Edelman et al. 1987), phosphorylated *Drosophila* DNA topoisomerase II; two to three phosphates are incorporated into each topoisomerase II polypeptide chain (Ackerman et al. 1985, 1988). In this study, no autophosphorylation of topoisomerase occurred, and serine was the only amino acid residue modified by casein kinase II. The reaction was moderately specific, showing an apparent K_m of 0.4 μM for topoisomerase II. Phosphorylation of the enzyme stimulated DNA relaxation activity approximately two- to threefold and was reversed by treatment with alkaline phosphatase.

Even more compelling is the evidence that casein kinase II type enzymes phosphorylate DNA topoisomerase I. The phosphorylation of Novikoff topoisomerase I increases enzymatic activity (Durban et al. 1983); the serine residue, which is the primary site of phosphorylation in vivo, is phosphorylated by purified casein kinase II in vitro (Durban et al. 1985). In *Xenopus* ovaries, phosphorylation of DNA topoisomerase I may be obligatory for enzymatic activity (Kaiserman et al. 1988). DNA topoisomerase activity was lost when the *Xenopus* enzyme was treated with phosphatase and was regained after treatment with ATP and a casein-kinase-II-like activity (Fig. 1). Indeed, dephosphorylation blocked the topoisomerase I nicking of DNA induced by the drug camptothecin. Thus, phosphorylation of this enzyme appears to be required for the formation of the initial covalent enzyme/DNA complex. Whether this total dependence on phosphorylation for catalytic activity is a special property of DNA topoisomerase from *Xenopus* ovaries or whether it is characteristic of most DNA topoisomerase I enzymes remains to be established.

Protein kinase C and a calmodulin-dependent protein kinase also phosphorylated topoisomerase II in vitro (Sahyoun et al. 1986). Protein kinase C is a well-characterized calcium-dependent and phospholipid-activated protein kinase. This cellular enzyme is the receptor for phorbol esters and tumor promoters with structural similarities to diacylglycerol.

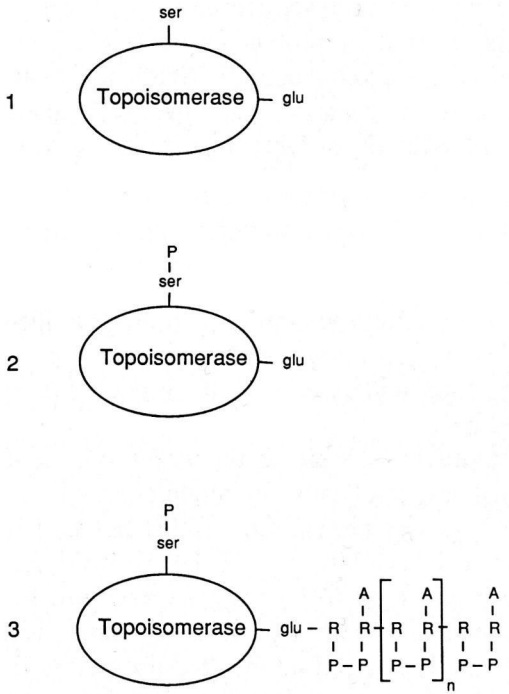

Figure 1 Some possible covalent modifications of DNA topoisomerases in higher eukaryotes. (*1*) Unmodified topoisomerase. (*2*) Topoisomerase phosphorylated at a serine residue. (*3*) Topoisomerase with both a phosphoserine and a poly(ADP-ribose) chain. A indicates adenine, R indicates ribose, P indicates phosphate. Type I and II topoisomerases can be modified by serine kinases, such as casein kinase II, to yield a phosphorylated enzyme as shown in structure 2. In many cases, this modification stimulates activity and in *Xenopus* oocytes phosphorylation of serine may be required for activity. The reversibility of phosphorylation has been documented in vitro but not in vivo; this reaction is presumably carried out by a protein phosphatase. Topoisomerases are also substrates for poly(ADP-ribose) synthetase in higher eukaryotes (structure *3*). The modification site on the protein is probably a glutamate residue, and ADP-ribose chains of varying lengths are added to the enzyme. The DNA breaking-joining activity of the modified proteins becomes progressively more inhibited as the chains become longer. This process is reversed by a second nuclear enzyme, poly(ADP-ribose) glycohydrolase, which reactivates the modified enzyme.

Protein kinase C shows a high affinity for its topoisomerase target with an apparent K_m for *Drosophila* topoisomerase II of about 100 nM. The reaction requires calcium and phosphatidyl serine and is stimulated by phorbol esters. Approximately 1 mole of phosphate is incorporated per mole of topoisomerase II, suggesting that a single site on the enzyme

might be phosphorylated. Like the studies described above with casein kinase II, topoisomerase phosphorylated by protein kinase C exhibited two- to threefold stimulation of DNA-relaxing activity. Studies of intact cells of the sponge *Geodia cydonium* are consistent with the involvement of protein kinase C in the phosphorylation of DNA topoisomerase II in vivo (Rottmann et al. 1987).

B. Other Phosphorylation Reactions

Other examples of topoisomerase phosphorylation have been described only in vitro. These include phosphorylation at tyrosine residues and the automodification of prokaryotic topoisomerases with the precise site of phosphorylation presently undefined.

Tyrosine protein kinase activity is intrinsic to numerous oncogene products and cell receptors that are implicated in modulation of cell growth. There are two reports of tyrosine protein kinases that can modify topoisomerases (Tse-Dinh et al. 1984, Goldberg et al. 1985). Rous sarcoma virus transforming gene product $pp60^{src}$ phosphorylates tyrosine moieties on *Escherichia coli* topoisomerase I, *Micrococcus luteus gyrA* subunit, calf thymus topoisomerase I, and calf thymus topoisomerase II. In the cases of the type-I topoisomerases of *E. coli* and calf thymus, phosphorylation of tyrosine residues is associated with loss of greater than 90% of the DNA-relaxing activity. This result indicates that the tyrosine involved in coupled DNA breakage and reunion activity may be a target of protein kinase modification. Similar results were obtained with a less well-defined 75-kD cellular protein kinase isolated from uninfected cells. However, since these kinases are normally membrane-bound proteins that may not travel to the nucleus, the significance of these results awaits further testing.

Prokaryotic DNA topoisomerases have autophosphorylation activity. Three subunits that make up the bacteriophage T4 DNA topoisomerase complex are encoded by products of genes *39*, *52*, and *60* (see Huang, this volume). Gene *39* has been cloned (Huang 1986a) and overproduced in uninfected *E. coli* cells. It has a strong ATP-binding site and intrinsic protein kinase activity (W.M. Huang, unpubl.). The gp39 subunit carries out an autophosphorylation reaction and also modifies gp52 and gp60 of the T4 enzyme. gp52 has significant homology with *gyrA* (Huang 1986b), and the A and B subunits of DNA gyrase are substrates for modification by the T4 DNA topoisomerase gp39 protein kinase activity.

The T4 gp39 and *E. coli gyrB* protein have regions of distinct protein homology, and the B subunit of gyrase also has a kinase activity that is

weak by comparison with T4 gp39; *gyrB* protein undergoes an autophosphorylation reaction that is stimulated by Ca^{++} (W.M. Huang, unpubl.). The *gyrB* kinase activity also phosphorylates the *gyrA* subunit of *E. coli* gyrase. A major issue to be resolved is whether these protein kinase activities exhibited by DNA topoisomerase II subunits are a manifestation of the intrinsic catalytic mechanism in which ATP is cleaved as DNA strand passage occurs or, alternatively, serve some regulatory role.

C. Poly(ADP-ribosylation) of DNA Topoisomerases

The poly(ADP-ribosylation) of DNA topoisomerases has been shown to occur efficiently in vitro (Ferro et al. 1983; Jongstra-Bilen et al. 1983; Ferro and Olivera 1984; Darby et al. 1985). This modification is carried out by a nuclear enzyme, poly(ADP-ribose) synthetase, which has been extensively characterized in several eukaryotic systems (for reviews, see Hayaishi and Ueda 1982; Ferro and Olivera 1987; Ueda 1987). Nicotinamide-adenine dinucleotide (NAD) is the substrate for all ADP-ribosylation reactions. The glycosidic bond between the nicotinamide ring and ribose (a high-energy bond) is cleaved, and the ADP-ribose moiety is transferred to the DNA topoisomerase target (probably to a carboxyl group of a glutamate side chain although this has not been directly established for DNA topoisomerases). The synthetase then continues transferring ADP-ribose residues to form ribose-ribose glycosidic linkages so that a chain of ADP-ribose monomer units (poly[ADP-ribose]) is covalently attached to the DNA topoisomerase (see Fig. 1). An unusual property of the modifying enzyme in vivo is that the synthetase is quiescent without an activating DNA structure. Interruptions in the DNA double helix, including double-strand breaks and single-strand nicks, are absolutely required to activate poly(ADP-ribose) synthetase. In the absence of activating DNA, the modification is quickly removed by another nuclear enzyme, poly(ADP-ribose) glycohydrolase.

The human gene for poly(ADP-ribose) polymerase (huADPRP) has been cloned, sequenced, and mapped (Cherney et al. 1987; Kurosaki et al. 1987; Uchida et al. 1987). Human and mouse proteins are closely related evolutionarily, and domains for an NAD-binding site, an automodification segment, and a DNA-binding domain containing two "zinc fingers" have been identified (Mazen et al. 1989). In a mouse, this gene is on chromosome 1, closely linked to the autoimmune locus *gld* (*g*eneralized *l*ymphoproliferative *d*isorder) (Huppi et al. 1989).

In vitro, when DNA topoisomerase I from calf thymus is extensively modified by the homologous poly(ADPR-synthetase), DNA-relaxing activity is abolished (Ferro et al. 1983; Jongstra-Bilen et al.1983; Ferro and

Olivera 1984). DNA topoisomerase II can also be modified (Darby et al. 1985). It has been suggested that poly(ADP-ribosylation) inhibits the DNA topoisomerase activity because the poly(ADP-ribose) chains decrease the affinity of topoisomerases for DNA. Since each ADP-ribose monomer has two negative charges, strong electrostatic repulsions exist between these moieties and the DNA substrate for topoisomerases (Ferro et al. 1984). Consistent with this electrostatic repulsion model (see Ferro and Olivera 1982; Zahradka and Ebisuzaki 1982; Ferro et al. 1984), the extent of modification that can be carried out by poly(ADPR-synthetase) in vitro depends on ionic strength. At low ionic strength, relatively minimal levels of modification can be carried out before the enzyme can no longer bind DNA. As the ionic strength increases, much more extensive modification occurs (up to 40 ADP-ribose residues/protein).

DNA topoisomerase I is one of the best exogenous acceptor proteins for ADPR-synthetase. Only a small subset of targets, including DNA topoisomerases, are efficiently modified under physiological conditions in vitro. The modification has also been detected in vivo (Krupitza and Cerutti 1989). The biological consequences of poly(ADP-ribose)-synthetase-inhibition include a variety of effects on DNA repair and an elevation of the frequency of sister chromatid exchanges (Oikawa et al. 1980).

In the absence of any exogenous protein, the synthetase carries out an automodification reaction and becomes so extensively poly(ADP-ribosylated) that it no longer binds DNA and therefore becomes inactive. However, exogenous targets such as DNA topoisomerase I depress automodification by competing with the synthetase as a substrate. The poly(ADP-ribosylation) of the synthetase itself may serve as a "timing device" so each enzyme molecule catalyzes poly(ADP-ribosylation) at a DNA strand break for a limited period of time before the enzyme self-inactivates.

II. PERSPECTIVES

The experimental data described in this chapter suggest that the covalent modification of topoisomerases may play a regulatory role in vivo. DNA topoisomerases of higher eukaryotes are substrates for protein kinases and poly(ADPR-synthetase) in vitro and have been identified in the phosphorylated and ADP-ribosylated states in vivo.

The report that *Xenopus* DNA topoisomerase I requires phosphorylation for enzymatic activity indicates that in some vertebrate systems, the degree of phosphorylation may be the critical factor determining the

amount of intracellular DNA topoisomerase activity. The question that needs to be addressed is how widespread is the requirement that DNA topoisomerases be phosphorylated in order to be active? Are the *Xenopus* results restricted to certain stages in the life cycle of vertebrates, or will this be a general feature of topoisomerases? The fact that yeast topoisomerase I can be cloned and expressed in *E. coli* in an active form (Bjornsti and Wang 1987) is indicative that this enzyme may be active without being phosphorylated.

In contrast with the stimulatory effects of serine phosphorylation, tyrosine phosphorylation and poly(ADP-ribosylation) are potential mechanisms for inactivating topoisomerases. However, the tyrosine kinases that have been studied to date are not nuclear proteins, and therefore the in vivo significance of tyrosine modification remains problematic. In contrast, poly(ADP-ribose) synthetase is a ubiquitous nuclear enzyme in higher eukaryotes; the evidence that this modification has physiological relevance is more compelling.

Phosphorylation and ADP-ribosylation are not specific to DNA topoisomerases; the best characterized enzymes relevant to DNA topoisomerase modification, casein kinase II and poly(ADP-ribose) synthetase, are known to modify a number of other proteins under physiological conditions. It seems likely that these enzymes are general triggers for changing the physiological state of the nucleus. The cellular function of these DNA topoisomerase modifications is best explored in the larger context of synthetase and casein kinase II physiology, and the entire spectrum of proteins modified needs to be considered.

Casein kinase II may be involved in cellular growth control. The enzyme appears to be activated when cells are proliferating. In contrast, poly(ADP-ribosylation) may be a general nuclear response to unscheduled interruptions in the DNA double helix such as strand breaks due to DNA damage. When the synthetase becomes activated, the enzyme modifies a number of proteins involved in the chromatin structure including histones and topoisomerases.

Thus, changes in the chromatin structure and metabolism may take place in the vicinity of a DNA strand break as a consequence of chromosomal protein ADP-ribosylation. Since sister chromatid exchange increases when synthetase is inhibited in vivo, it was previously suggested that poly(ADP-ribosylation) of topoisomerase may be part of a general mechanism of suppressing mitotic recombination events at DNA strand breaks. Such a mechanism would be important for long-lived multicellular organisms in order to decrease the probability that accumulated recessive mutations become homozygous. Clear evidence has been published recently that the activity of topoisomerases directly affect the fre-

quency of crossing-over events (Christman et al. 1988; Kim and Wang 1989; Wallis et al. 1989).

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REFERENCES

Ackerman, P., C.V.C. Glover, and N. Osheroff. 1985. Phosphorylation of DNA topoisomerase II by casein kinase II: Modulation of eukaryotic topoisomerase II activity *in vitro*. *Proc. Natl. Acad. Sci.* **82:** 3164–3168.

———. 1988. Phosphorylation of DNA topoisomerase II *in vivo* and in total homogenates of *Drosophila* Kc cells. The role of casein kinase II. *J. Biol. Chem.* **263:** 12653–12660.

Bjornsti, M. and J.C. Wang. 1987. Expression of DNA topoisomerase I can complement a conditional-lethal topoisomerase I mutation in *E. coli*. *Proc. Natl. Acad. Sci.* **84:** 8971–8975.

Cherney, B.W., O.W. McBride, D. Chen, H. Alkhatib, K. Bhatia, P. Hensley, and M.E. Smulson. 1987. cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci.* **84:** 8370–8374.

Christman, M.F., F.S. Deitrich, and G.R. Fink. 1988. Mitotic recombination in the rDNA of *S. cerevesiae* is suppressed by the combined action of topoisomerases I and II. *Cell* **55:** 413–425.

Darby, M.K., B. Schmitt, J. Jongstra-Bilen, and H.P. Vosberg. 1985. Inhibition of calf thymus type II DNA topoisomerase by poly ADP-ribosylation. *EMBO J.* **4:** 2129–2134.

Durban, E., M. Goodenough, J. Mills, and H. Busch. 1985. Topoisomerase I phosphorylation *in vitro* and in rapidly growing Novikoff hepatoma cells. *EMBO J.* **4:** 2921–2926.

Durban, E., J.S. Mills, D. Roll, and H. Busch. 1983. Phosphorylation of purified Novikoff hepatoma topoisomerase I. *Biochem. Biophys. Res. Commun.* **111:** 897–905.

Durban, E., D. Roll, G. Beckner, and H. Busch. 1981. Purification and characterization of a nuclear DNA binding phosphoprotein in fetal and tumor tissues. *Cancer Res.* **41:** 537–545.

Edelman, A.M., D.K. Blumenthal, and E.G. Krebs. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56:** 567–613.

Ferro, A.M. and B.M. Olivera. 1982. Poly (ADP-ribosylation) *in vitro*. Reaction parameters and enzyme mechanism. *J. Biol. Chem.* **257:** 7808–7813.

———. 1984. Poly (ADP-ribosylation) of DNA topoisomerase I from calf thymus. *J. Biol. Chem.* **259:** 547–559.

———. 1987. Intracellular pyridine nucleotide degradation and turnover. In *Pyridine nucleotide coenzymes* (ed. D. Dolphin et al.), p. 25–77. Wiley, New York.

Ferro, A.M., N.P. Higgins, and B.M. Olivera. 1983. Poly (ADP-ribosylation) of a DNA topoisomerase. *J. Biol. Chem.* **258:** 6000–6003.

Ferro, A.M., L.H. Thompson, and B.M. Olivera. 1984. Poly (ADP-ribosylation) and DNA topoisomerase I in different cell lines. In *Proteins involved in DNA replication* (ed. U. Hubscher and S. Spadari), p. 441–447. Plenum Press, New York.

Goldberg, A.R., T.W. Wong, and Y. Tse-Dinh. 1985. Properties of the major species of tyrosine protein kinase in rat liver: Effect on DNA topoisomerase activity. *Cancer Cells* **3:** 369–379.

Hayaishi, O. and K. Ueda, eds. 1982. *ADP-ribosylation reactions*, p. 674. Academic Press, New York.

Huang, W.M. 1986a. Nucleotide sequence of a type II DNA topoisomerase gene. Bacteriophage T4 gene 39. *Nucleic Acids. Res.* **14:** 7751–7765.

———. 1986b. The 52-protein subunit of T4 DNA topoisomerase is homologous to the *gyr*A protein of gyrase. *Nucleic Acids Res.* **14:** 7379–739O.

Huppi, K., K. Bhatia, D. Siwarski, D. Klinman, B. Cherney, and M. Smulson. 1989. Sequence and organization of the mouse poly (ADP-ribose) polymerase gene. *Nucleic Acids Res.* **9:** 3387–3401.

Jongstra-Bilen, J., M.-E. Ittel, C. Niedergang, H.-P. Vosberg, and P. Mandel. 1983. DNA topoisomerase from calf thymus is inhibited *in vitro* by poly (ADP-ribosylation). *Eur. J. Biochem.* **136:** 391–396.

Kaiserman, H.B., T.S. Ingebritsen, and R.M. Benbow. 1988. Regulation of *Xenopus laevis* DNA topoisomerase I activity by phosphorylation *in vitro*. *Biochemistry* **27:** 3216–3222.

Kim, R.A. and J.C. Wang. 1989. A subthreshold level of DNA topoisomerases leads to the excision of yeast rDNA as extrachromosomal rings. *Cell* **57:** 975–985.

Krupitza, G. and P. Cerutti. 1989. ADP-ribosylation of ADPR-transferase and topoisomerase I in intact mouse epidermal cells JB6. *Biochemistry* **28:** 2034–2040.

Kurosaki, T., H. Ushiro, N. Mitsuuchi, K. Kangawa, H. Matsuo, T. Hirose, S. Inayama, and Y. Shizuta. 1987. Primary structure of human poly (ADP-ribose) synthetase as deduced from cDNA sequence. *J. Biol. Chem.* **262:** 15990–15997.

Mazen, A., J. Menissier-deMurcia, M. Molinete, F. Simonin, G. Gradwohl, G. Poirier, and G. deMurcia. 1989. Poly (ADP-ribose) polymerase: A novel finger protein. *Nucleic Acids Res.* **17:** 4689–4698.

Mills, J.S., H. Busch, and E. Durban. 1982. Purification of a protein kinase from human Namalwa cells that phosphorylates topoisomerase I. *Biochem. Biophys. Res. Commun.* **109:** 1222–1227.

Oikawa, A., H. Tohda, M. Kanai, M. Miwa, and T. Sugimura. 1980. Inhibitors of poly (ADP-ribose) polymerase induce sister chromatid exchanges. *Biochem. Biophys. Res. Commun.* **97:** 1311–1316.

Rottmann, M., H.C. Schröder, M. Gramzow, K. Renneisen, B. Kurelec, A. Dorn, U. Friese, and W.E.G. Müller. 1987. Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester. Role of protein kinase C in the phosphorylation of DNA topoisomerase II. *EMBO J.* **6:** 3939–3944.

Sahyoun, N., M. Wolf, J. Besterman, T. Hsieh, M. Sander, H. Levine, K. Chang, and Cuatrecasas. 1986. Protein kinase C phosphorylates topoisomerase II: Topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci.* **83:** 1603–1607.

Sander, M., J.M. Nolan, and T. Hsieh. 1984. A protein kinase activity tightly associated with *Drosophila* type II DNA topoisomerase. *Proc. Natl. Acad. Sci.* **81:** 6938–6942.

Tse-Dinh, Y., T. Wous, and A.R. Goldberg. 1984. Virus- and cell-encoded tyrosine protein kinases inactivate DNA topoisomerases *in vitro*. *Nature* **312:** 785–786.

Uchida, K., T. Morita, T. Sato, T. Ogura, R. Yamashita, S. Noguchi, H. Suzuki, H. Nyunaya, M. Miwa, and T. Sugimura. 1987. Nucleotide sequence of a full-length cDNA for human fibroblast poly (ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* **148:** 617–622.

Ueda, K. 1987. ADP-ribosylation of proteins. Enzymology and biological significance. *Mol. Biol. Biochem. Biophys.* **137:** 1–237.

Wallis, J.W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein. 1989. A hyper recombination mutation in *S. cerevesiae* identifies a novel eukaryotic topoisomerase. *Cell* **58:** 409–419.

Zahradka, P. and K. Ebisuzaki. 1982. A shuttle mechanism for DNA-protein interactions. *Eur. J. Biochem.* **127:** 579–585.