Identification of Fructose 3-Phosphate in the Lens of Diabetic Rats

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Fructose 3-phosphate, a novel monosaccharide phosphate, has been identified in the lens of diabetic rats. This compound, which is not present in normal lenses, is a protein glycosylating agent and enzyme inactivator. In addition, because of its structural features, this metabolite is relatively labile and undergoes hydrolysis to yield inorganic phosphate and the potent glycosylating agent, 3-deoxyglucosone. The increase in the concentration of fructose 3-phosphate in the lens of diabetic rats suggests that it and its hydrolysis product, 3-deoxyglucosone, may be responsible in part for the development of some diabetic complications in the lens.

ALTHOUGH A CAUSAL CONNECTION between hyperglycemia and the pleiotropic complications of diabetes mellitus has been established, the detailed sequence of events resulting from this condition and leading to the observed functional deficits remains to be elucidated (*1*). Several mechanisms for the biochemical basis of diabetic complications have been proposed: the osmotic stress hypothesis (*2*), the *myo*-inositol depletion hypothesis (*3*), the Na^+- and K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) inactivation hypothesis (*4*), the glycosylation hypothesis (*5*), and a recent proposal involving enhanced de novo synthesis of diacylglycerol and activation of protein kinase C (*6*). None of these proposed mechanisms has proven entirely satisfactory, and recent data suggests that osmotic stress and *myo*-inositol depletion may not be the primary mechanisms of diabetic complications in humans (*7*). The hypothesis most strongly supported by data from human subjects is the glycosylation hypothesis (*8*), which states that diabetic complications are caused, at least in part, by protein modification and cross-linking by glucose and glucose-derived metabolites. This nonenzymatic process occurs in two stages (*5*). In stage one, glucose reacts with an amine group of a protein to form a Schiff's base, which then rearranges to the more stable Amadori product (*9*). These reactions are slow (equilibrium is reached after 4 to 6 weeks) and reversible, so that a reduction in the concentration of glucose will lead to a decrease in the amount of glycosylated protein. The reactions of the second stage are irreversible, however, leading to the accumulation of fluorescent yellow material (*10*) similar to that associated with aging and nonenzymatic browning of food proteins (*11*). In agreement with this mechanism, long-lived proteins such as collagen and lens crystallins from diabetic subjects are significantly more glycosylated than those from age-matched normal controls (*8*).

We have now obtained evidence in support of a variant of the glycosylation hypothesis that invokes a novel monosaccharide, fructose 3-phosphate (F3P), and its breakdown product, 3-deoxyglucosone (3dG), as glycosylating agents. The first evidence that new metabolites may be present in the lens of diabetic animals came from ^{31}P nuclear magnetic resonance (NMR) spectroscopic examination of rabbit lenses that had been incubated in medium with a high glucose concentration and of lenses from diabetic rats (*12, 13*). These experiments showed the presence in the ^{31}P-NMR spectra of two unidentified compounds resonating at 6.5 and 5.8 ppm (Fig. 1A). In normal lenses these compounds were either totally absent (5.8 ppm) or present in much lower concentrations (6.5 ppm) (*14*).

Recently, we purified one of these metabolites (6.5 ppm) and identified it as sorbitol 3-phosphate (S3P) (*15*). Although the second metabolite proved more difficult to isolate because of its relative lability, the kinetics of its accumulation in the lens of diabetic rats and its response to the aldose reductase inhibitor Sorbinil (*13*) suggested a precursor-product relation with S3P. This possibility was supported by similarities in pK_a between the two compounds (5.4 for S3P and 5.5 for the 5.8-ppm compound) and the fact that both compounds appear as doublets in the ^1H-coupled ^{31}P-NMR spectrum. To test this relation, we synthesized the two likely precursors of S3P, glucose 3-phosphate and F3P and compared their ^{31}P-NMR spectra with those of extracts of lenses from diabetic rats. Synthetic F3P was found to coresonate exactly with the 5.8-ppm peak (Fig. 1, A and B). This overlap in resonances was absolute and independent of *p*H (Fig. 1, C and D). In addition, both the 5.8-ppm resonance in the extract and synthetic F3P were shown to be alkali labile and to produce S3P and mannitol 3-phosphate after reduction with $NaBH_4$. The chances for a random independent coincidence of such properties are extremely small; we have therefore attributed the 5.8-ppm resonance to F3P.

Synthetic F3P used in the identification was prepared from fructose (*16*) and characterized by thin-layer chromatography (TLC), elemental analysis, and ^{31}P- and ^{13}C-NMR spectroscopy (Fig. 2). From the relative peak intensities in these spectra, we calculated the relative proportions of the various anomeric forms of this ester in solution (Table 1) and found them to be similar to those of fructose and other fructose phosphomonoesters. The major difference between F3P and these compounds was the high proportion of the open chain carbonyl,

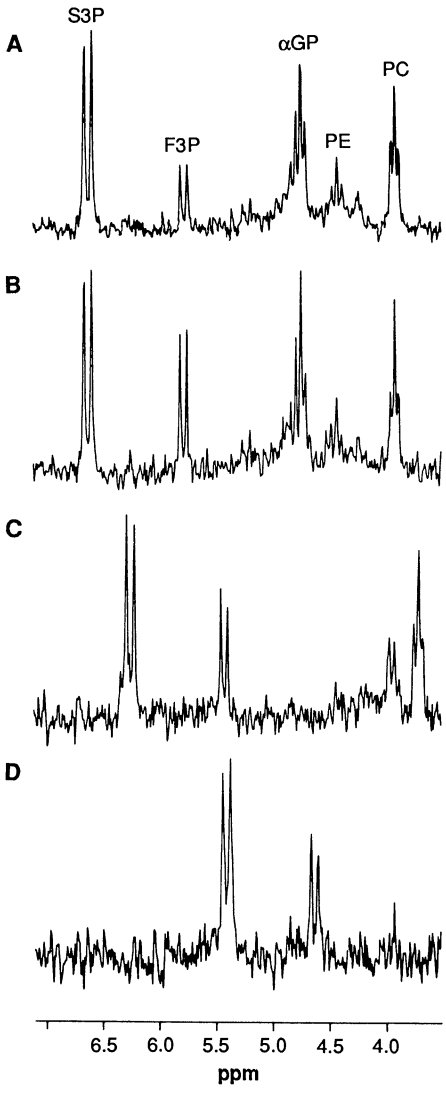

Fig. 1. (**A**) The phosphate monoester region of a ^1H-coupled ^{31}P-NMR spectrum (*31*) of a perchlorate extract (*p*H 7.2) from five rat lenses 3 weeks after the induction of diabetes (*32*). Peak identities are S3P, sorbitol 3-phosphate; F3P, fructose 3-phosphate; αGP, α-glycerolphosphate; PE, phosphorylethanolamine; and PC, phosphorylcholine. (**B**) Extract used in (A) after addition of 85 μ*M* synthetic F3P (*p*H 7.2). (**C**) Sample as in (B), except at *p*H 6.4. (**D**) Sample as in (B), except at *p*H 5.6.

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Table 1. Relative proportions of anomeric forms of fructose and F3P in solution at 25°C.

Anomer	Fructose* (%)	F3P† (%)
β-Pyranose	75	62.3 ± 0.6
α-Pyranose	Trace	2.5 ± 0.3
β-Furanose	21	19.8 ± 0.4
α-Furanose	4	9.7 ± 0.4
Open chain	0.7	5.0 ± 0.3
Open-chain hydrate		0.7 ± 0.2

*From Angyal and Bethell (*17*). †Relative proportions of anomers (means ± SEM, n = 3) were determined from ^{13}C- and ^{31}P-NMR spectra of synthetic F3P by the use of the PIQABLE automatic quantification program (*30*).

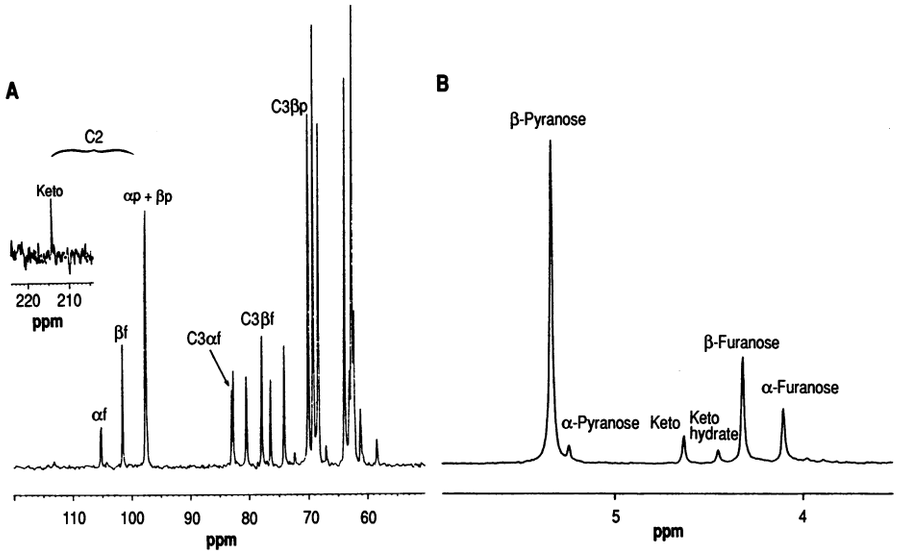

Fig. 2. (**A**) ^{1}H-decoupled, natural abundance ^{13}C-NMR spectrum of F3P. Peak identities are based on the anomeric assignments of fructose (*33*); αf and βf, α- and β-furanose; αp + βp, α- and β-pyranose. The anomeric distribution is most clearly seen in the C2 resonances, which include the peaks at 95 to 105 ppm and the keto peak at 214 ppm. The C3 peaks identified in the spectrum are those that have shifted downfield from their position in the ^{13}C-NMR spectrum of fructose. The purity of the preparation was confirmed by the fact that when the integral of the C2 peaks is set to 1, the remaining peaks integrate to a value of 5, as expected for a six-carbon compound. (**B**) ^{1}H-decoupled ^{31}P-NMR spectrum of synthetic F3P. Peaks were identified by their relative intensity, expected relative chemical shift position, and secondary peak splitting (*34*).

which at 5.0% is higher than in fructose (0.7%) or the other fructose phosphomonoesters (2.0%) (*17*). This result suggested that F3P may function as a glycosylating agent because the reactivity of reducing sugars as glycosylating agents is determined by the percentage of the open chain form (*18*). In addition, phosphate esters of such compounds, with a phosphate group proximal to the carbonyl, are quite labile, generating α-dicarbonyl compounds on hydrolysis. For example, hydrolysis of dihydroxyacetone phosphate (DHAP) or glyceraldehyde phosphate (GAP) leads to the formation of methylglyoxal (*19*), whereas the hydrolysis of F3P yields 3dG (*20*). Both these α-dicarbonyls are toxic to animals and to cultured cells (*21*), presumably, in part, because of their glycosylating activity (*22*).

As demonstrated by a number of groups, 3dG can be produced from glucose and fructose upon incubation of these sugars with proteins. These studies also suggest that this α-dicarbonyl may be the active intermediate in the fructose- and glucose-mediated cross-linking of proteins (*23*). To compare the glycosylating potential of F3P with these previously studied saccharides, we incubated lysozyme with various concentrations of these four compounds at 37°C for 28 days. The reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and by size-exclusion high-performance liquid chromatography (HPLC). In contrast to glucose and fructose, which show little or no reactivity in these assays, F3P appears to be a potent glycosylating and cross-linking agent, equal to or greater than 3dG in its reactivity (Fig. 3). In fact, at the lowest concentration studied (1 m*M*), F3P was about twice as effective as 3dG.

Glycosylation and cross-linking of protein in cells, in addition to producing structural alterations, should lead to enzyme inactivation, especially in tissues with low protein turnover such as the lens. As such inactivation has been demonstrated with a number of α-dicarbonyls (*22*), we examined the effect of 3dG and F3P on enzymatic activity by incubating these sugars with the enzymes lactate dehydrogenase and fructose 1,6-bisphosphate aldolase. After 24 hours of incubation, 3dG produced an almost total inactivation of both enzymes whereas F3P was more selective, producing a partial inactivation of aldolase (Table 2). In contrast to 3dG and F3P, both glucose and fructose had no effect on activity of the two enzymes. The enzyme-inactivating capacities of F3P and its hydrolysis product 3dG suggest that these metabolites may be responsible, in part, for the losses of enzymatic activity reported in the lens of diabetic rats (*24*).

The only compounds structurally similar to F3P, which have been previously described in living tissues, are DHAP and GAP, which arise in the course of normal glycolysis. Control of the potential toxicity of these compounds is accomplished by their rapid conversion to more stable metabolites by relevant enzymes such as GAP dehydrogenase [found in the human liver at a concentration of ~5 μ*M* (*25*)] as well as by conversion of their reactive breakdown product, methylglyoxal (*19*), to lactate by

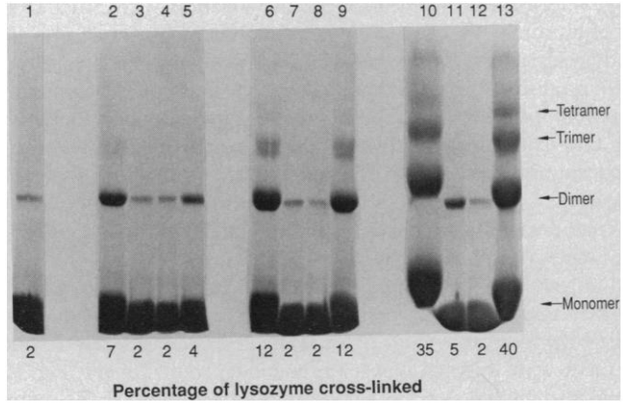

Fig. 3. SDS-PAGE of lysozyme after incubation at 37°C at a concentration of 25 mg/ml in physiological buffer for 4 weeks in the absence of substrate (lane 1) or with F3P (lanes 2, 6, and 10), fructose (lanes 3, 7, and 11), glucose (lanes 4, 8, and 12), or 3dG (lanes 5, 9, and 13) at concentrations of 1 m*M* (lanes 2 to 5), 10 m*M* (lanes 6 to 9), or 100 m*M* (lanes 10 to 13). The mean percentages of cross-linked protein, as estimated by densitometry and HPLC analysis are indicated below each lane (SEM values were ≤5% of means, n = 4). The incubation buffer consisted of 120 m*M* KCl, 20 m*M* NaCl, 1 m*M* $MgCl_2$, and 20 m*M* inorganic phosphate (*p*H 7.25). Samples were fractionated on a 10% slab gel and stained with Coomassie blue. The lysozyme monomer is at the dye front of the gel, and the positions of multimers are shown. The glycosylated monomer migrates behind the unmodified monomer.

Table 2. Effect of F3P and 3dG on enzyme activities. Enzymes were incubated with 100 m*M* substrate at 37°C for 24 hours. The incubation buffer consisted of 120 m*M* KCl, 20 m*M* NaCl, 1 m*M* $MgCl_2$, 20 m*M* inorganic phosphate (*p*H 7.25), 0.1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* dithiothreitol, and 0.1% NaN_3. Three incubations were performed, and enzyme assays were done in duplicate. Results shown represent a mean of the remaining activity as a percentage of the maximal velocity and SEM values were ≤5% of the means.

Enzyme	Remaining activity (%)				
	Control	Glucose	Fructose	F3P	3dG
Lactate dehydrogenase	91	91	92	95	15
Fructose 1,6-bisphosphate aldolase	95	100	95	70	5

the glyoxalase system (*26*). These reactions keep the concentrations of DHAP and GAP in cells low, typically at 10 to 20 nmol per gram of wet weight (*27*). In contrast, analysis of the ^{31}P-NMR spectra of lens from diabetic rats shows that the steady-state concentration of F3P is ~500 nmol per gram of wet weight.

The total glycosylation potential of F3P in the lens of diabetic rats can be estimated from the kinetic data on the accumulation of F3P and S3P in this tissue (*13*). After induction of diabetes, there is a rapid accumulation of F3P, the content increasing from 0 to 500 nmol per gram of wet weight in the first 24 hours. After this initial period, the concentration of F3P remains constant, whereas the accumulation of S3P continues for at least 2 weeks at a rate of 250 nmol per gram of wet weight per day. If we assume that the initial rate of F3P production remains constant, then there must be sink for F3P to account for the discrepancy of 250 nmol per gram of wet weight per day between the rates of production of the two metabolites. One likely sink may be protein glycosylation. Whereas the figure of 250 nmol per gram of wet weight per day is an upper limit, it is more than sufficient to account for the glycosylation and cross-linking observed in the lens of diabetic animals (*28*).

Although the details of the biosynthesis of F3P are still unknown, evidence from both in vivo and in vitro experiments (*13*) suggests that this compound is formed from glucose. Once formed, F3P can follow one of at least three metabolic routes: (i) reduction to S3P (presumably by an aldose reductase); (ii) reaction with proteins; or (iii) hydrolysis to 3dG. As the hydrolysis product of F3P, 3dG, is a potent glycosylating agent, accumulation of F3P in the lens can lead to protein glycosylation by two routes. The relative contributions of these two mechanisms will depend on the concentration of 3dG, which in turn is a function of the rate of hydrolysis of F3P and the efficiency of any detoxification mechanisms for 3dG. In contrast to methylglyoxal, 3dG does not appear to be a substrate of the glyoxalase system (*29*).

To determine if the findings in the rat lens could be extended to other tissues affected by diabetic complications, we examined extracts from sciatic nerves of normal and diabetic rats by ^{31}P-NMR for the presence of F3P and S3P. In the normal tissue, neither compound was detected (<2 nmol per gram of wet weight). In diabetic tissue, however, after both 6 weeks and 3 months of diabetes, sciatic nerves show 10 ± 5 nmol per gram of wet weight of F3P but lack S3P.

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16. Fructose 3-phosphate was synthesized from fructose in three steps. Fructose was reacted with four equivalents of cyclohexanone and a catalytic amount of H_2SO_4 to produce a blocked fructose 1,2:4,5-di-O-cyclohexylidene-D-fructopyranoside (*33*). This material was then phosphorylated at the free 3-hydroxyl group with a 10% excess of diphenyl phosphorochloridate in pyridine [D. Brown, F. Hayes, A. Todd, *Berichte* **90**, 936 (1957)], and the product was purified by silica gel chromatography. The phenyl groups were removed from the phosphate by hydrogenolysis in ethyl acetate over platinum at 14 psi of H_2 for 24 hours, and, after removal of catalyst and solvent, the residue was treated with 90% trifluoroacetic acid for 5 min at 23°C. The resulting F3P was purified by ion-exchange chromatography on Dowex-1 (Cl) by means of a linear gradient of NaCl (0 to 0.4*M*). Appropriate fractions (as assayed by ^{31}P-NMR) were concentrated, desalted on a Sephadex G-10 gel filtration column, and lyophilized to a white powder (28% yield). This powder decomposed at 122° to 125°C, and had a specific rotation, $[\alpha]_D^{23}$, of −36° (at a concentration of 2 g per 100 ml of H_2O).
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28. From the data of Abraham *et al.* [E. C. Abraham, M. S. Swamy, R. E. Perry, in *The Maillard Reaction in Aging, Diabetes and Nutrition*, J. W. Baynes and V. M. Monnier, Eds. (Liss, New York, 1989), pp. 123–139], we calculate the total protein concentration in the lens to be 150 mg/ml. Taking the relative abundance of arginine in the lens to be 4% (*21*), the concentration of arginine in the lens can be estimated at 30 μmol per gram of wet weight. If we assume that most of the glycosylation observed in the lens is due to modification of arginine residues, the 0.25% per day rate of glycosylation of proteins in the lens of diabetic rats [Abraham *et al.*, above] would be equivalent to modification of 75 nmol of arginine per gram. Thus the apparent rate of consumption of F3P (250 nmol per gram of wet weight) is more than sufficient to account for all of the glycosylation observed.
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31. NMR data was acquired on a Bruker AM-400

31. NMR data was acquired on a Bruker AM-400 spectrometer at 162 MHz with a 1.2-ml microcell in a 10-mm ^{31}P probe in 5000 scans with 60° pulses and a 1.4-s relaxation delay. Chemical shifts were referenced to an external standard of 85% orthophosphate at 0.0 ppm or to an internal standard of glycerophosphorylcholine set at 0.49 ppm.
32. Diabetes was induced in rats (Sprague-Dawley, 200 g of body weight) by a single tail vein injection of streptozotocin (65 mg per kilogram of body weight) in 100 m*M* citrate buffer (*p*H 4.5). Diabetes was confirmed by monitoring urine glucose, water consumption, and urine output.
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35. We thank I. Rose, F. Matchinsky, and C. Marano for discussions and S. Osman, A. Goll, and S. Singh for technical assistance. Supported in part by NIH grants EY-08223, CA-06927, and CA-41078 and by the Commonwealth of Pennsylvania.

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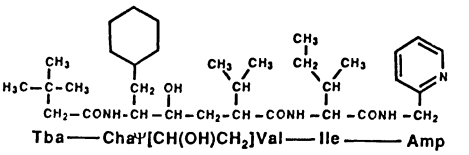

Fig. 1. Chemical structure of U-81749 (Tba-ChaΨ[CH(OH)CH_2]Val-Ile-Amp); see text for abbreviations. All stereocenters are of the *S* configuration.

A Synthetic HIV-1 Protease Inhibitor with Antiviral Activity Arrests HIV-Like Particle Maturation

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A synthetic peptidemimetic substrate of the human immunodeficiency virus 1 (HIV-1) protease with a nonhydrolyzable pseudodipeptidyl insert at the protease cleavage site was prepared. The peptide U-81749 inhibited recombinant HIV-1 protease in vitro (inhibition constant K_i of 70 nanomolar) and HIV-1 replication in human peripheral blood lymphocytes (inhibitory concentration IC_{50} of 0.1 to 1 micromolar). Moreover, 10 micromolar concentrations of U-81749 significantly inhibited proteolysis of the HIV-1 gag polyprotein (p55) to the mature viral structural proteins p24 and p17 in cells infected with a recombinant vaccinia virus expressing the HIV-1 *gag-pol* genes. The HIV-1 like particles released from inhibitor-treated cells contained almost exclusively p55 and other gag precursors, but not p24. Incubation of HIV-like particles recovered from drug-treated cultures in drug-free medium indicated that inhibition of p55 proteolysis was at least partially reversible, suggesting that U-81749 was present within the particles.

The protease encoded by HIV-1 is required for the processing of the viral polyproteins encoded by the *gag* and *pol* genes into mature virion proteins (*1–3*). This processing involves cleavage of the gag precursor (p55) to form the four structural proteins of the virion core (p17, p24, p8, and p7); in addition, processing of the p160 gag-pol precursor yields these structural proteins as well as enzymes essential for HIV replication [that is, protease and reverse transcriptase, and integration protein (*4*)]. The HIV-1 protease is an aspartic protease that is enzymatically active upon dimerization (*5–11*).

To date the major efforts to identify synthetic compounds combating HIV have focused principally on compounds targeting the viral reverse transcriptase or its cellular receptor, the CD4 molecule (*12–15*). Relatively little information has been reported on compounds specifically designed to inhibit the HIV-1 protease or describing their anti-HIV activities. Therefore we prepared a variety of peptidemimetic HIV-1 protease inhibitors and evaluated their anti-HIV properties. These inhibitors contain a hydroxyethylene isostere (Ψ[CH(OH)CH_2]) as a nonhydrolyzable, synthetic replacement of the scissile amide bond at the P_1-P_1' site. Such chemical modifications have been shown recently by Richards *et al.* (*16*) and Tomasselli *et al.* (*17*) to be key chemical determinants of highly potent inhibitors (such as H-261 and U-85548E) of the protease in vitro. However, we determined experimentally that a direct correlation between protease inhibition in vitro and anti-HIV activity in cellular systems could not be established (that is, U-85548E and H-261–like derivatives do not inhibit HIV replication in cell culture).

We investigated the structure-activity relations of HIV-1 protease inhibitors of the formula W-XaaΨ[CH(OH)CH_2]Yaa-Ile-Z that varied in amino- and carboxyl-terminal functionalization as well as the P_1-P_1' amino acids. On the basis of this strategy we produced U-81749, which contained an amino-terminal *tert*-butylacetyl, a P_1 cyclohexylalanine (Cha), a P_1' Val, and carboxyl-terminal aminomethylpyridine (Tba-ChaΨ-[CH(OH)CH_2]Val-Ile-Amp; Fig. 1). Recombinant HIV-1 protease was competitively inhibited by U-81749 in vitro [K_i = 70 n*M* (*17*)]. Good selectivity for the HIV-1 enzyme was maintained compared with human renin (>100 times lower potency toward renin). Because of U-81749's enhanced aqueous solubility compared to other synthetic gag analogs prepared, we investigated its anti-HIV and protease inhibitory properties in cellular systems.

We evaluated the anti-HIV activity of U-81749 by determining the levels of HIV p24 and HIV RNA in culture supernatants of human peripheral blood lymphocytes (PBLs) 3 and 4 days after HIV infection (*18*). Addition of 1 μ*M* U-81749, a level determined to be nontoxic to PBL proliferation (*19*), to the culture medium immediately after virus addition reduced the levels of HIV p24 ~70% compared with those found in the supernatants of control, infected cells (8 versus 25 and 18 versus 58 ng/ml of p24 at 3 and 4 days after infection, respectively). However, the levels of HIV RNA detected in supernatants from drug-treated cultures were only ~3 pg/ml of RNA at these times compared with control values of 91 and 278 pg/ml of RNA. In the presence of 0.1 μ*M* drug the levels of HIV p24 and RNA were still approximately 25 to 50% lower than those in control supernatants (19 versus 25 and 25 versus 58 ng/ml of p24 at 3 and 4 days after infection, respectively); at lower concentrations of U-81749 (0.001 to 0.01 μ*M*), no significant inhibition was seen.

To investigate whether the HIV protease was the actual target of U-81749 in cells, we studied processing of the HIV p55 and the maturation of HIV-like particles in recombinant virus–infected CV-1 cells (Figs. 2 and 3). As recently described by Karacostas *et al.* (*20*), infection of CV-1 cells with a recombinant vaccinia virus (vVK-1) engineered to express the HIV *gag-pol* genes results in the synthesis and processing of gag-pol precursors. Initially, the most prominent HIV polypeptides synthesized are p55, p46, and p41; with time, however, these proteins are further processed to the mature viral pro-

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