Biochemical and Biophysical Research Communications **275,** 26–32 (2000)
doi:10.1006/bbrc.2000.3251, available online at http://www.idealibrary.com on IDEAL®

Phosphorylation of AZT-Resistant Human Immunodeficiency Virus Type 1 Reverse Transcriptase by Casein Kinase II *in Vitro:* Effects on Inhibitor Sensitivity

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Received July 11, 2000

Casein kinase II (CKII) phosphorylates wild-type (WT) recombinant reverse transcriptase (RT) mainly in the p66 subunit *in vitro.* Phosphorylation of T215F RT and D67N/K70R/T215F/K219Q RT (AZT-resistant RT) *in vitro* increases discrimination against AZTTP 2.5- and 3.6-fold, respectively. This *in vitro* resistance can be reversed by treatment of phosphorylated AZT-resistant RT with phosphatase. Phosphorylation has no effect on WT RT. Terminal transferase activity of RT is selectively suppressed on phosphorylated AZT-resistant RT. Resistance to phosphonoformic acid (PFA, foscarnet) increases 3-fold upon phosphorylation of AZT-resistant RT. Although T215, the most important residue for AZT-resistance, is part of a CKII consensus target site, serines are primarily phosphorylated relative to threonines. Mutational analysis shows that phosphorylation can be reduced to 10% that of WT when amino-acid changes are introduced both in the "fingers" subdomain and motif D. These results suggest that phosphorylation of RT might be one factor involved in drug resistance *in vivo.*

Key Words: **drug-resistance; AZT; mechanism; nucleotide analogue; polymerase.**

Efficient chemotherapy directed against the human immunodeficiency virus (HIV) is impeded by the gradual loss of potency of nucleoside drugs. The essential viral reverse transcriptase (RT), encoded by the *pol* gene, is the target of such drugs. Mutations accumulating in the *pol* gene specify variant RTs able to confer a drug-resistant phenotype to a recombinant virus assayed in infected cells. However, when a purified variant RT is assayed *in vitro,* a loss of affinity of this RT for the nucleotide analogue does not always account clearly for the observed resistance. For example, when AZT is given as the sole drug, HIV-1 genotypes show a typical pattern of amino-acid substitutions involving 4 to 6 residues at position 41, 67, 70, 210, 215, and 219. Viruses carrying D67N/K70R/T215F/K219Q changes in RT are >100-fold resistant to AZT relative to wild-type viruses. However, when the biochemical properties of purified D67N/K70R/T215F/K219Q RT are examined *in vitro,* a single biochemical parameter cannot unambiguously account for this >100-fold resistance (1–4). Amongst distinct biochemical properties of D67N/K70R/T215F/K219Q RT are the discrimination of AZT-TP against the natural substrate dTTP about 1- to 4-fold relative to wild-type (1, 4–7), an increased processivity (8), and an increased binding to the AZT-MP terminated DNA primer (9) consistent with an increased pyrophosphorolytic/polyphosphate-synthesis repair of the terminal analogue (7, 10).

Given that molecular mechanisms of RT-mediated AZT-resistance might involve an uncharacterized cellular factor (4), it was of interest to test if known protein kinases could phosphorylate RT *in vitro* and modify or potentiate one of the biochemical properties listed above. During the course of this study, phosphorylation of RT was reported *in vitro* and using cellular extracts, and found to stimulate RT activity about 2-fold (11–14). Therefore, we investigated the putative CKII phosphorylation sites in RT as well as whether or not this 2-fold stimulation in RT activity could also be involved in AZT-resistance.

Abbreviations used: CKII, casein kinase II; RT, reverse transcriptase; AZT-TP, 3′-deoxy 3′-azido deoxythymidine 5′-triphosphate; AZT-MP, 3′-deoxy 3′-azido deoxythymidine 5′-monophosphate; TLE, thin layer electrophoresis.

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0006-291X/00 $35.00

EXPERIMENTAL PROCEDURES

Construction of mutant pol genes. The plasmid p66RT7 was used as a starting point to construct mutant *pol* genes as described (6). Briefly, synthetic oligonucleotides corresponding to the coding sense and antisense nucleotide sequence of a fragment of RT were designed so as to introduce the desired mutation and carry ligatable ends reconstituting a silent restriction site. They were then phosphorylated using polynucleotide kinase and ATP, annealed one to each other, and ligated into digested and dephosphorylated p66RT7 DNA, an RT expression vector (6). Introduction of the mutation was verified by nucleotide sequencing.

Purification of recombinant wild-type and variant RT. The plasmid p66RT7 was used to express wild-type RT in *E. coli* XL1-Blue cells. Recombinant RT (either wild-type of variant) carrying a $(His)_6$ tag was purified using phosphocellulose chromatography followed by Nickel-Agarose chromatography (Qiagen) as described (6). The purification of RT that does not carry a $(His)_6$ tag was as described (9).

Phosphorylation of RT by protein kinases in vitro. A panel a immunoprecipitated kinases was tested for their ability to phosphorylate RT *in vitro.* Histone H1 was used as a positive control. Phosphorylation of RT (10–50 μg) by CKII was performed using 10–100 units of CKII and 15 to 150 μM γ-^{32}P-ATP during 1 h at 30°C. Control experiments showed that the maximal amount of radiolabel incorporated into RT was reached after 15 min incubation and stayed constant afterwards. To quantitate the amount of incorporated radioactivity, the gel was dried, and quantitation of band products was achieved using photostimulatable plates and a FujiImager. Autophosphorylated CKII served as an internal control for quantitation. CKII was from New England Biolabs.

Purification of phosphorylated RT. Phosphorylated RT (50 μg) in the CKII/ATP reaction mixture was further separated from ATP and CKII by absorption onto Nickel-Agarose beads, elution using 0.2 M imidazole, and dialysis against the initial RT storage buffer (50% glycerol, 25 mM KPO4 pH 7.4, 1 mM DTT, 0.5 mM EDTA). The final yield was around 45%.

Phosphoamino-acid analysis. Phosphorylated proteins were separated by SDS-PAGE on 10% gels. Excised band were digested in gel slices by 2 μg of trypsin (Promega) as described (15), yielding about 97% of the initial radioactivity of the band. The mixture was further hydrolysed by 5.7 M HCl at 110°C for 90 min, dried under vacuum, and subjected to thin layer electrophoresis (TLE) using phosphoamino-acid internal standards and cellulose plates (Merck, 20 × 20 cm, 0.1 μm). TLE was bidimensional as described (16). Dried plates were developed using ninhydrin and autoradiography.

DNA polymerase assays. Reverse transcription was assayed as described (6). DNA-dependent DNA polymerase assays were performed using a primer/template system made of a 21-primer (5′-ATACTTTAACCATATGTACC-3′) annealed to a 35-mer (5′-GGTCCGTTGC**A**TGCGGATACATATGGTTAAAGTAT-3′) DNA template. The single template "A" specifying a single thymidine insertion site four bases away from the 3′-end of the primer is shown in bold. RT (50 nM) was absorbed onto the primer/template (100 nM) in RT buffer, and reaction was initiated by the addition of nucleotide: inhibitor:$MgCl_2$ mix as described in figure legends. Reaction aliquots were quenched at indicated times by formamide-containing buffer, and the mixture was analysed by denaturing gel electrophoresis followed by autoradiography. Quantitation of band products was achieved using photostimulatable plates and a FujiImager.

RESULTS

Screening protein kinases for their ability to phosphorylate RT in vitro. Since most *in vitro* studies of RT use an *E. coli* produced enzyme, such an enzyme might lack a post-transcriptional modification occuring only in eukaryotic cells. Phosphorylation on serine/threonine residues is such a modification. Therefore, several eukaryotic cellular kinases were tested for their ability to phosphorylate pure recombinant RT *in vitro.* CKII was found to incorporate radioactive phosphate from γ-^{32}P-ATP into the p66 subunit of RT (Fig. 1, lane 4) while the p51 subunit alone was essentially untouched (Fig. 1, lane 1; lanes 5–8 are discussed below under "*Mutational analysis of putative CKII phosphorylation sites*"). The level of incorporation of radioactive phosphate into RT was independent from the presence of the histidine tag in C-terminus (not shown). None of the other tested kinases could phosphorylate RT (cdc2, cdk1, 2, 4, 5, and 6, not shown). This result is consistent with the report of Harada *et al.* showing that CKII-phosphorylated RT stimulated RT activity (13). CKII is a serine/threonine kinase of broad specificity and tissue distribution. In order to determine if this stimulation is related to drug-resistance, nucleotide selectivity was measured using phosphorylated RT in comparison with their unphosphorylated counterparts.

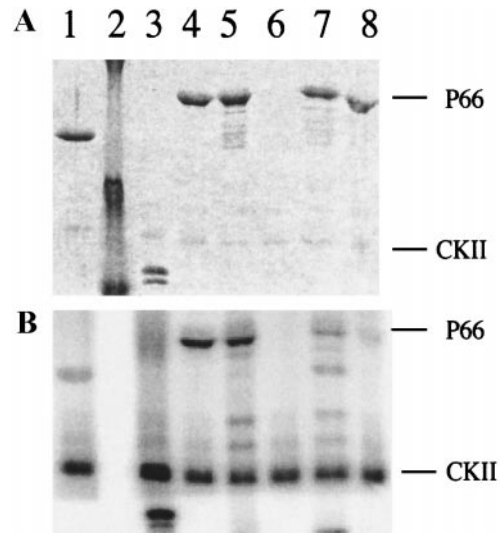

FIG. 1. Phosphorylation of RT by CKII *in vitro.* (A) 10% SDS-PAGE analysis of various purified RT (3 μg per lane). The gel was stained using coomassie blue. Reactions in all lanes except lane 2 contained CKII and radiolabeled ATP. Lane 1: p51 subunit; lane 2: MW markers (75, 45, and 30 kD); lane 3: histone H1; lane 4: WT-p66 subunit; lane 5: T215F-p66 subunit; lane 6: control without RT; lane 7: K220Q-p66 subunit; lane 8: D67N/K70R/T216F/K220Q-p66 subunit. (B) Autoradiograph of the gel shown in panel A. The gel was dried, exposed, and quantitated using an image plate and a FujiImager.

Discrimination against AZT-TP by phosphorylated RT. Two RT bearing the clinically relevant AZT-resistance amino-acid changes T215F and D67N/K70R/T215F/K219Q were prepared. CKII-mediated phosphorylation indicated that these two RTs could be

TABLE 1

Inhibition of DNA Polymerization by Phosphorylated RT Using AZT-TP

RT	IC_{50} (nM)[c]
WT	18 ± 0.7
WT-P[a]	20 ± 1
Fold increase	1
T215F	26 ± 1.8
T215F-P[a]	65 ± 5.5
Fold increase	2.5
AZT-Res	31.5 ± 1.5
AZT-Res-P[a]	115 ± 8
AZT-Res-P/CIAP[b]	30 ± 2.5
Fold increase	3.6

[a] WT-P, T215F-P, and AZT-Res-P indicate the phosphorylated forms of WT, T215, and AZT-res RT, respectively.

[b] AZT-Res-P/CIAP indicated phosphorylated RT that has been treated with calf intestine alkaline phosphatase.

[c] Poly(rA)/oligo(dT)$_{18-21}$ as the primer/template system.

labelled at a level equal to about 2/3 that of WT RT (this is described later in Table 3). After phosphorylation by CKII *in vitro,* they were assayed for reverse transcription in the presence of dTTP and its competitive inhibitor for chain-termination AZT-TP. The results are presented in Table 1. Phosphorylation of WT RT had no effect on AZT-TP discrimination, whereas phosphorylation of T215F RT yielded a 2.5-fold increase in IC_{50}. The effect of phosphorylation was more pronounced for RT bearing the four amino-acid changes: phosphorylation of AZT-resistant RT enhanced AZT-TP discrimination 3.6-fold as judged by the increase in IC_{50} relative to the unphosphorylated AZT-resistant RT. Treatment of phosphorylated AZT-resistant RT with phosphatase reversed the discrimination back to the value observed with unphosphorylated AZT-resistant RT, indicating that the observed effect was indeed due to phosphorylation of RT by CKII. In total, the increase in IC_{50} from WT to phosphorylated RT equaled 6.4-fold (from 18 nM to 115 nM, Table 1).

To confirm these results, the effects of phosphorylation of RT were also assayed using a ^{32}P-labelled primer extension and gel assay. In this type of experiment, DNA polymerization by RT is allowed to proceed in the presence of dA, dG, dC nucleotides and a mixture of dTTP and AZT-TP. When a "dA" is present in the template, RT inserts either a dT-MP or an AZT-MP depending on the concentration ratio of the latter two nucleotides. When AZT-MP is inserted, DNA polymerization terminates. Analysis of the reaction products by denaturing gel electrophoresis shows that a band product shorter than full extension product and corresponding to the AZT-MP terminated DNA chain appears on the autoradiogram of the gel. The abundance of this band product relative to extension products greater or equal in size than the AZT-MP terminated DNA inversely reflects the discrimination property of RT against AZT-TP: the higher intensity the lower discrimination against AZT-TP.

RTs bearing clinically relevant amino-acid changes were phosphorylated by CKII, and compared relative to their unphosphorylated form using this AZT-TP discrimination test. The results are shown in Fig. 2. WT RT discriminated AZT-TP to the same extend as phosphorylated WT RT (Fig. 2A) as judged by the same intensity of both the "T" band product and the 35 and 36 mer band products. For phosphorylated AZT-resistant RT, however, not only the intensity of the "T" band product was lower but also the total intensity of all band products past the "T" band was higher than for its unphosphorylated counterpart (Fig. 2B). PhosphorImager analysis allowed to measure a 2-fold better discrimination of AZT-TP by phosphorylated AZT-resistant RT than with its unphosphorylated counterpart, a result consistent with our homopolymeric template/primer assay. We conclude that phosphorylation of RT by CKII induces a modest change in AZT-TP selection relative to the natural substrate dTTP.

Additional unexpected information could be obtained from this gel assay. HIV RT is known to possess a terminal transferase activity by which a nucleotide is added at the 3′-terminus of a blunt double stranded DNA (17). This activity can be seen in Fig. 2 (and Fig. 3, see below) where an additional dNMP is added in a template-independent fashion, yielding a 36-mer prod-

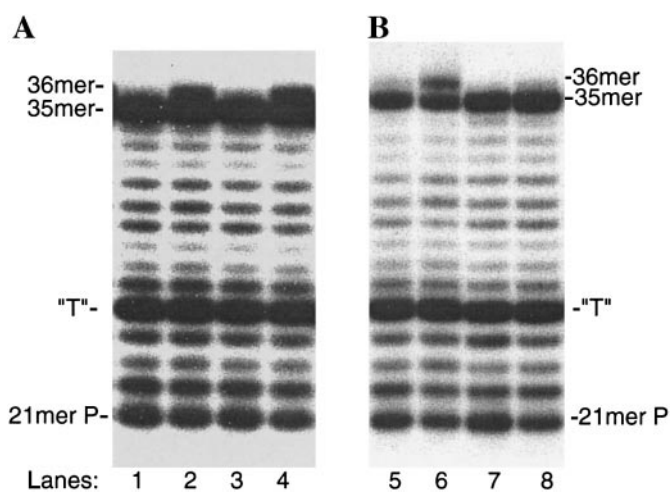

FIG. 2. DNA polymerization by phosphorylated RT. A 5′-^{32}P-labeled 21-primer was annealed to a 35-mer DNA template (100 nM) specifying a single thymidine insertion site (indicated "T") four bases away from the 3′-end of the primer. Polymerization was initiated by the addition of RT (50 nM), nucleotides (50 μM of each dATP, dCTP, dGTP, dTTP, and 50 μM AZT-TP) for 5 min. (odd lanes) and 20 min. (even lanes) at 37°C. Aliquots were withdrawn during the time course of the reaction, and products analysed by denaturing gel electrophoresis. (A) WT-(lanes 1, 2) and phosphorylated WT RT (lanes 3, 4). (B) AZT-resistant (lanes 5, 6) and phosphorylated AZT-resistant RT (lanes 7, 8).

uct from a 35-mer template. Phosphorylation of WT RT had no effect on this terminal transferase activity, whereas the latter was suppressed when AZT-resistant RT was used (compare lane 6 to lane 8 in both Fig. 2B and Fig. 3B). Interestingly, the suppression of the terminal transferase activity indicates that other biochemical properties of RT might also be altered by phosphorylation. This prompted us to examine pyrophosphorolysis of AZT-MP terminated primers.

Pyrophosphorolytic repair of AZT-MP terminated primer by phosphorylated RT. AZT-resistance by D67N/K70R/T215F/K219Q RT is believed to be due in part to an increased repair of the AZT-MP terminated primer by pyrophosphorolysis and/or ATP-mediated unblocking mechanism (7, 9, 10, 18). Pyrophosphorolysis is the reversal of the polymerization reaction where a pyrophosphate (PPi) molecule attacks the AZT-MP blocked chain to yield AZT-TP and a free 3′-OH group. Consequently, PPi has an IC_{50} of about 1 mM on the polymerization reaction by WT RT (7). When PPi inhibition is tested using D67N/K70R/T215F/K219Q RT, the IC_{50} decreases to 0.4 mM (7).

The same gel assay as for discrimination was used to measure the influence of RT phosphorylation on pyrophosphorolysis. In this assay, an AZT-MP terminated DNA chain is first produced using RT and the same primer/template as described above. The pyrophosphorolysis reaction is then started by the addition of pyrophosphate and dTTP. After binding PPi, RT removes the terminal AZT-MP to yield AZT-TP and a free 3′-OH end, and RT is able to use either AZT-TP to yield again a terminated product or dTTP to yield a extension product greater in size than the AZT-MP terminated DNA chain. As a consequence, the band product corresponding to "T" on the gel is disappearing over time (Fig. 3). The results are presented in Fig. 3. Whereas phosphorylation had no effect on WT RT (Fig. 3A, lanes 1–4), phosphorylated AZT-resistant RT was resistant to PPi-mediated repair of the blocked DNA (Fig. 3B, compare lanes 5 and 6 with lanes 7 and 8: the intensity of the "T" band product is much lower in lane 6 than in lane 8).

Phosphonoformic acid (PFA) is an analogue of PPi and a potent inhibitor of several viral polymerases. When assayed on AZT-resistant RT, the IC_{50} of PFA decreases 2-fold relative to WT RT, a result that is consistent with the involvement of pyrophosphorolysis in this type of drug-resistance (7, 18). It was thus of interest to determine if phosphorylation of RT by CKII could also influence PPi or PFA inhibition as it does with AZT-TP. The inhibition by PPi is difficult to assay using homopolymeric primer/template because of the precipitation of PPi in the presence of Mg^{++}. Therefore, the IC_{50} of PFA was determined for non-phosphorylated and phosphorylated AZT-resistant RT as described above. Phosphorylation had no effects on the IC_{50} of

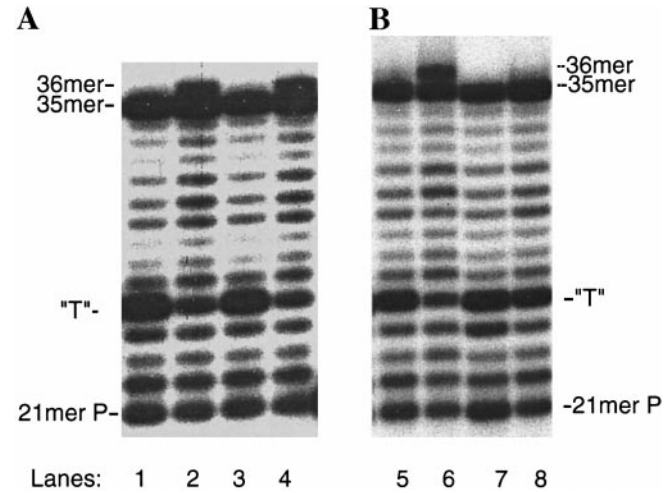

FIG. 3. Pyrophosphorolytic repair of incorporated AZT-MP by phosphorylated RT. Same experimental design as in Fig. 2, except that reactions were performed in the presence of 500 μM pyrophosphate. Over time, the amount of products larger than 25 nucleotides increases in the presence of PP_i with a concommitant decrease of the "T" band product due to pyrophosphorolytic repair of the analogue-terminated DNA chain followed by DNA polymerization. (A) WT- (lanes 1, 2) and phosphorylated WT RT (lanes 3, 4). (B) AZT-resistant (lanes 5, 6) and phosphorylated AZT-resistant RT (lanes 7, 8).

WT RT (not shown). However, the IC_{50} of AZT-resistant RT was measured at 0.27 ± 0.06 μM and increased up to 0.9 ± 0.17 μM when this RT was phosphorylated. This 3-fold increase in IC_{50} for PFA is in agreement with results obtained using the gel assay shown above. We conclude that phosphorylation of RT by CKII also alters the traffic of pyrophosphate at the RT active site.

Phosphoamino-acid analysis of phosphorylated RT. Phosphorylation of RT by CKII alters biochemical properties related to DNA polymerization. Thus, we expected that serine/threonine residues in the vicinity of the polymerase active site might be the target of CKII, and potential target sites were examined. WT RT and T215F were phosphorylated using CKII *in vitro,* and each mixture was separated using SDS-PAGE electrophoresis. After staining with coomassie blue, the RT band was cut off the gel and subjected to protease digestion followed by total acid hydrolysis. Phosphoamino-acids were identified by bidimensional chromatography and autoradiography (Fig. 4). Approximately 95% of the radioactivity was found on a spot corresponding to phosphoserine, and 5% on a spot corresponding to phosphothreonine. T215F RT was consistently less phosphorylated than WT RT (see below), and this difference was tentatively attributed to serine residues. As shown in Fig. 4, the spot corresponding to labelled phosphoserines is weaker for T215F RT than for WT RT.

Mutational analysis of putative CKII phosphorylation sites. In order to assess how RT activity could be modulated by phosphorylation, potential CKII phos-

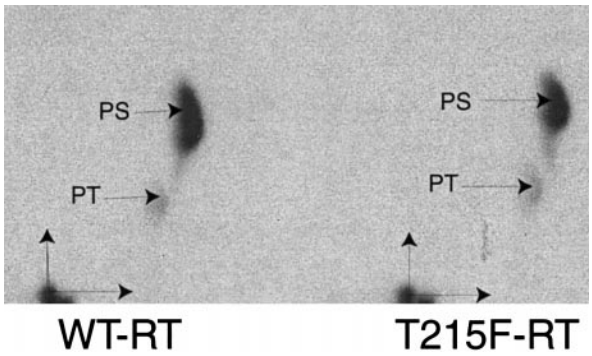

FIG. 4. Phosphoamino-acid analysis of phosphorylated RT. Bidimensional TLE using radioactively labelled phosphoamino-acid, internal standards, and cellulose plates. The dried plate was developed using ninhydrin. This is an autoradiograph of the plate. Arrows at right angles indicate the 2 dimensions of migration. PT and PS designate phosphothreonine and phosphoserine, respectively. (A) WT RT. (B) T215F RT.

phorylation sites were searched along the RT gene using the PROSCAN algorythm (19). Nine putative sites were found. Their position, amino-acid sequence, and their spatial distribution on the RT crystal structure are reported in Table 2. We found that two of the putative phosphorylation sites were in the vicinity (less than 3 amino-acids away) of residues involved in AZT-resistance. They are T39 and T215 (Table 2). As stated in the introduction, T215F/Y is a key mutation involved in AZT-resistance. Examination of the RT crystal structure (20) shows that T215 belongs to motif D, a short loop exposed to the solvent. Motif D encompasses amino-acids 213 to 223, and is located at the entrance of the nucleotide channel (6). It is thus of interest to determine if T215F eliminates a CKII phosphorylation site either directly or indirectly. However, as described above, T215F alone does not totally account for the increased AZT-TP discrimination: the presence of all four mutations is required (Table 1).

TABLE 2

Consensus Sequences for Phosphorylation by CKII in RT

Residues in RT	Consensus: S/T-X-X-D/E	AZT resistance[a]	Exposition to the solvent[b]
S3	SPIE	−	±
T39	TEME	+	+
T107	TVLD	−	−
S191	SDLE	−	±
T200	TKIE	−	+
T215	TTPD	+	+
T253	TVND	−	+
S489	SGLE	−	−
S513	SESE	−	±

[a] Sites bearing at least one amino-acid change involved in AZT-resistance are indicated with +.

[b] Sites having both S/T and D/E exposed to the solvent are marked +, those having only one residue are marked ±, and buried sites are marked −.

TABLE 3

Phosphorylation and Reverse Transcriptase Activity of Variant Relative to WT RT

RT	Phosphorylation relative to WT[a] (%)	RT activity relative to WT[b]
WT	100	++
T215F	65	++
D67N, K70R, T215F, K219Q (AZT-Res)	66	++
D67N, K70R	66	++
D218N	52	++
K220Q	49	++
D67N, K70R, T216F, K220Q	10	+

[a] Mean of two independent experiments, values are less than 5% different.

[b] ++: 80 to 100% of WT; +: 40 to 60% of WT, using poly(rA)/oligo$(dT)_{18-21}$ as the primer/template system.

To characterize the determinants of increased AZT-TP discrimination induced by both phosphorylation and the four mutations, a series of RT genes mutated in these regions was constructed (Table 3). The corresponding RTs were purified and phosphorylated by CKII using radiolabeled ATP. The reaction was allowed to go to completion, the amount of total radioactive phosphate incorporated into RT was measured and expressed relative to phosphorylated WT RT (Table 3). All RT bearing the amino-acid change T215F incorporated about one third less radioactive label than WT RT. In particular, the AZT-resistant RT bearing the four substitutions was not labelled to a lower extent than T215F RT. One straightforward explanation was that T215F eliminated phosphorylation of T215, but this hypothesis is not consistent with the fact that phosphoamino-acid analysis pinpointed a difference in phosphoserine content between WT and T215F RT. Therefore, we conclude that T215F, located in motif D of RT, has an indirect effect on the phosphorylation status of RT. In other words, T215F might alter the general conformation of the RT active site in such a way that phosphorylation of other residues (mainly serine as judged by the phosphoamino-acid analysis) would be altered.

To determine if the conformation of motif D is critical for phosphorylation of RT, another series of RT genes mutated in motif D and its surrounding was constructed (Table 3). K220Q and D218N greatly reduced (up to 50%) the degree of phosphorylation of RT, indicating that motif D is indeed critical to the phosphorylation of RT by CKII (Table 3 and Fig. 1). In the crystal structure of RT, motif D is facing the "fingers" subdomain made of $\beta 3/\beta 4$ strands. The latter strands encompass amino-acids 60 to 75 and thus carry the D67N and K70R AZT-resistance changes. D67N/K70R

RT incorporated about one third less of radioactive phosphate than WT RT, indicating that the conformation of the nucleotide binding site as a whole is indeed critical for phosphorylation by CKII. When changes concerning both $\beta 3/\beta 4$ strands and motif D such as D67N/K70R/T216F/K220Q were combined, the corresponding RT could barely be phosphorylated by CKII (Fig. 1, lane 8). Although this RT is fully active upon single nucleotide incorporation, its DNA synthesis specific activity is about 40% as that of wild-type under processive conditions using homopolymeric primer/templates (Table 3, and not shown).

We also tested if the presence of a DNA-primed RNA- or DNA template bound to RT could influence the pattern of phosphorylation of RT by CKII, but no difference was observed whether or not any primer/template system was present in the reaction mixture. Also, replacing T39 by either alanine or aspartate had no effect either on the phosphorylation degree of RT or on AZT-TP discrimination. Phosphothreonine or phosphoserine residues can often be replaced by an acidic residue that mimicks phosphorylation to yield a protein which is biochemically undistinguishable from the authentic phosphorylated protein. This was not the case here as T39D/D67N/K70R/T215F/K219Q was biochemically undistinguishable in any respect from the AZT-resistant D67N/K70R/T215F/K219Q RT (not shown). The same experiment was performed with S3A and S513A but no difference was observed relative to WT RT. S513 is located close to the RNase H domain, and its phosphorylation could have an indirect effect on the DNA polymerization properties of RT. However, neither phosphorylated or non-phosphorylated S513A nor S513D RTs showed any effect on reverse transcription.

DISCUSSION

The mechanistic understanding of AZT-resistance has been lagging behind the determination of the genetic causes of this viral drug-resistance. Several authors have reported that the high level of drug-resistance observed *in vivo* (about 120-fold) does not correlate to drug-resistance observed using purified RT *in vitro* (1- to 4-fold) (1, 4–7). The lack of correlation has been tentatively attributed to an unknown cellular factor. However, most studies have been conducted using RT expressed and purified from *E. coli* cultures. It is well known that *E. coli* does not provide the same post-translational modifications to a recombinant protein as an eukaryotic cell does. Serine/threonine phosphorylation is such a modification, and being present in eukaryotic but not in *E. coli* cells, is indeed a good candidate to account for the *in vivo/in vitro* discrepancy. This observation lead us to assay various kinases for their ability to phosphorylate RT, and we were encouraged by recent studies reporting that CKII is able to stimulate RT activity (13).

The understanding of AZT-resistance in molecular terms has been recently improved by the proposition that an increased pyrophosphorolytic repair of the chain-terminating AZT-MP could be achieved by AZT-resistant RT (9). Subsequently, Arion *et al.* found that pyrophosphorolytic repair is indeed involved (7, 18), and Meyer *et al.* found that the β and γ phosphate of ATP could also provide the pyrophosphate moiety required to repair the AZT-MP terminated primer (10).

Our results show that phosphorylation of AZT-resistant RT but not WT RT increases discrimination against AZT-TP, a results consistent with phosphorylation being potentially the missing cellular factor. However, two results temperate this view. First, the effects of phosphorylation are modest (about 3-fold) and not sufficient to explain the remaining >40-fold difference between *in vivo* and *in vitro* data. Second, phosphorylated AZT-resistant RT is less able to repair an AZT-MP terminated primer than unphosphorylated AZT-resistant RT. An increased pyrophosphorolysis activity and/or ATP-mediated repair together with an increased sensitivity to PFA are hallmarks of AZT-resistance (7, 10, 18). It is shown here that the opposite is observed, *i.e.*, a decreased pyrophosphorolytic repair and hence a potential increase in AZT sensitivity when AZT-resistant RT is phosphorylated. It would be interesting to assay ATP-mediated repair using phosphorylated RT to see whether it is affected in the same manner as pyrophosphorolytic repair. Nevertheless, the effects observed here are interesting because they are specific to AZT-resistant RT. For example, the suppression of terminal transferase activity by CKII phosphorylation might have a biological relevance yet to be discovered.

Although precise assignment of phosphorylated residues remains to be done, our results indicate that it is safe to propose that serine residues are involved that do not belong to the consensus CKII target sequence S--D/E. It is tempting to speculate that phosphorylation of serine residues in the vicinity of the polymerase active site would alter either the movement of the motif D loop whose structure is important for nucleotide selectivity (6), or the movement of the “fingers” subdomain in which several drug-resistance mutation are found (3, 21). Alternately, partial or incomplete phosphorylation of threonine residues up to 5% as found in our phosphoamino-acid analysis might be responsible for the modest discriminational effect found here. This hypothesis awaits the isolation and direct identification of phosphorylated RT peptides, the mutational elimination of the phosphorylated residue, and the lack of distinct biochemical properties brought by the corresponding mutated RT.

ACKNOWLEDGMENTS

We thank Charles C. Richardson and Anne Debant for their help and encouragement, and Simon Sarfati and Catherine Gueirreiro for the synthesis and purification of AZT-TP. We also thank "Ensemble contre le Sida" for financial support.

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