Intracellular Substrates for the Primer-Unblocking Reaction by Human Immunodeficiency Virus Type 1 Reverse Transcriptase: Detection and Quantitation in Extracts from Quiescent- and Activated-Lymphocyte Subpopulations

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Human immunodeficiency virus type 1 (HIV-1) replication is inhibited by the incorporation of chain-terminating nucleotide analogs at the 3′ end of the nascent DNA chain during reverse transcription. Viral replication in the presence of these drugs results in selection of resistance mutations in the viral sequences encoding reverse transcriptase (RT), the replicative enzyme for HIV. Mutations at six codons in RT (M41L, D67N, K70R, L210W, T215Y or -F, and K219Q) are commonly selected by the nucleoside inhibitor 3′-azido-3′-deoxythymidine (AZT) and have been shown to confer AZT resistance through an increased ability to remove the chain terminator after it has been incorporated into DNA (1, 29, 46). These mutations are also correlated with significant resistance to other chain-terminating nucleosides (33, 34, 47).

Excision has been shown to occur by RT-catalyzed transfer of the chain-terminating residue to a variety of acceptor substrates in vitro by a reaction that is related to pyrophosphorolysis (1, 5, 16, 29, 31, 42, 43). The intracellular acceptor for this reaction is unknown, but likely candidates include nucleoside triphosphates and nucleoside diphosphates as well as inorganic pyrophosphate (PPi).

In this report, we show that cell extracts contain a mixture of acceptor substrates for the excision reaction. ATP or PPi, predominates depending on the cell type and its activation status. For these experiments, it was necessary to avoid errors in the measurement of PPi levels in the extracts, which can be affected by a number of factors: the breakdown of unstable intracellular compounds to form PPi during the extraction procedure, contaminating platelets, and cellular enzymes that alter PPi levels during the extraction process. Results obtained with an optimized extraction procedure disagree with widely quoted values of 130 to 150 μM for intracellular PPi concentration in unstimulated lymphocytes (3); our results are more in agreement with the data of De La Rosa et al. (8) that PPi concentrations are less than 10 μM. We also show that PPi concentrations increased to 55 to 79 μM in highly stimulated T cells. At this level of PPi, pyrophosphorolysis predominated and the overall rate of excision was similar for wild-type (WT) and AZT-resistant enzymes. To explain selection of AZT resistance mutations in vivo, it is possible that PPi-dependent excision is enhanced in the mutant virus but not detected in the in vitro assays. Alternatively, selection may occur in an intracellular environment that favors ATP-dependent excision over pyrophosphorolysis.

MATERIALS AND METHODS

Cells, cell subfractionation, and activation. The H9 T-lymphoid cell line was obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated) and 45 μg/ml gentamicin. Peripheral blood from healthy donors was obtained from Continental Blood Bank Service (Miami, FL), and the mononuclear cell fraction (PBMC) was isolated by centrifugation through Ficoll/Hypaque. Purified cell subpopulations were positively selected from the PBMC mixture by magnetic separation with anti-CD4, anti-CD8, or anti-CD14-conjugated microbeads (Miltenyi Biotec) to >96% purity as determined by flow cytometry. CD4+ and CD8+ T cells were either extracted immediately (unstimulated T cell extracts) or suspended at 106 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 10 units/ml interleukin 2 (Roche Diagnostics), 2 g/ml phytohemagglutinin (GIBCO BRL), and 45 μg/ml gentamicin, and incubated for 48 h before extraction (activated T-cell extracts). CD14+ cells were either extracted immediately (monocyte extracts) or induced to differentiate into macrophages by incubation for 7 days at 106 cells/ml in Macrophage-SFM medium (GIBCO BRL) supplemented with 500 units/ml of recombinant granulocyte-macrophage colony-stimulating factor (Immunex) in the absence of FBS and gentamicin. On day 7, the medium was replaced and the cells were cultured for an additional 48 h in the presence of 100 units/ml gamma interferon (PeproTech) and 100 ng/ml Escherichia coli lipopolysaccharide (Sigma). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

The activity of each cell subpopulation from each donor was determined by flow cytometry using monoclonal antibodies anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-CD14, or anti-CD69 conjugated to fluorochromes allophycocyanin, fluorescein isothiocyanate, phycoerythrin, or peridin chlorophyll protein (Becton Dickinson) in four-color flow cytometry assays. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson) with gating of viable cells.

Preparation of cell extracts. Cell extraction was similar to the procedure described by De La Rosa et al. (8), modified as described below. Cells were harvested by centrifugation, and cell pellets were resuspended in boiling water (75 μl/106 cells), incubated at 100°C for 70 s, and cooled in ice. After brief centrifugation, each extract was passed through a 3K MICROSEP microcentrator (Pall Gelman) to remove residual enzymatic activities, and the spin column was washed once with warm water. The filtrates were combined and lyophilized, reconstituted in 200 μl of Macrophage-SFM medium (GIBCO BRL) supplemented with 500 units/ml of recombinant granulocyte-macrophage colony-stimulating factor (Immunex) in the absence of FBS and gentamicin. On day 7, the medium was replaced and the cells were cultured for an additional 48 h in the presence of 100 units/ml gamma interferon (PeproTech) and 100 ng/ml Escherichia coli lipopolysaccharide (Sigma). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

The extraction step. Formation of PPi during boiling water or perchloric acid (monocyte extracts) or induced to differentiate into macrophages by incubation for 7 days at 106 cells/ml in Macrophage-SFM medium (GIBCO BRL) supplemented with 500 units/ml of recombinant granulocyte-macrophage colony-stimulating factor (Immunex) in the absence of FBS and gentamicin. On day 7, the medium was replaced and the cells were cultured for an additional 48 h in the presence of 100 units/ml gamma interferon (PeproTech) and 100 ng/ml Escherichia coli lipopolysaccharide (Sigma). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

The reaction mixture was transferred to a 96-well plate, and the reaction was stopped by boiling for 3 min. The 32P-labeled primer-template in the reaction mixture, the percentage converted to specific product, and the time of incubation. The rate of radioactive activity in [32P]dAMP and [32P]Ap4ddA, ddATP, GTP and ADP, respectively (Fig. 1B). The rate of Ap4ddA formation was approximately 3 times that of ddATP or [32P]Ap4ddA synthesis was plotted versus the concentration of added PPi, or ATP, respectively, and the linear portion of the curve was fit to the equation y = mx + b, where x and y are the concentrations of acceptor substrate (PPi or ATP) added and excision product (Ap4ddA or ddATP) formed, respectively. The concentration of PPi, or ATP in the cell extract is equal to b/m.

RESULTS

Acceptor substrates for excision of ddAMP by HIV-1 RT are present in H9 cell extracts. Incubation of [32P]dAMP-terminated DNA primer/template with HIV-1 RT(AZT) and H9 cell extract led to a time-dependent decrease in radioactive primer and increase in low-molecular-weight labeled products (Fig. 1A). These products were identified as Ap4ddA, ddATP, Gp4ddA, and Ap3ddA by their comigration with authentic substrates (data not shown) and resistance to digestion by calf intestinal phosphatase (Fig. 1A, lane 12). The identification of dinucleoside tetra- and triphosphates on the basis of electrophoretic mobility and sensitivity to enzyme digestion has been described previously (31). Ap4ddA, ddATP, Gp4ddA, and Ap3ddA correspond to products resulting from the transfer of [32P]ddAMP from the primer terminus to ATP, PPi, GTP and ADP, respectively (Fig. 1B). The rate of Ap4ddA formation was approximately 3 times that of ddATP or Gp4ddA and 10 times that of Ap3ddA.

The boiling-water extraction procedure was chosen after comparison with established extraction methods for measurement of nucleotide pools (2, 19, 23, 37, 48, 50) by adding [32P]dAMP or [32P]PPP to the cells at the time of excision and evaluating their recovery in the extract by gel electrophoresis (see Methods). After extraction for 16 h at −20°C with 70% methanol, the electrophoretic mobility of the [32P]dAMP was unaltered; however, after a third of the [32P]PPP, was cleaved to free phosphate, while the rest was incorporated into higher-molecular-weight species (data not shown). By contrast, greater than 97% of both compounds was unchanged after the boiling-water extraction followed by centrifugation through a 3K MICROSEP microconcentrator as described in Methods. Incubation of [32P]dAMP-terminated primer/template with
cell extract in the absence of HIV-1 RT<sup>AZT</sup> (Fig. 1A, lanes 1 to 3) or incubation with HIV-1 RT<sup>AZT</sup> in the absence of cell extract (lanes 4 to 6) resulted in the slow appearance of a phosphatase-sensitive compound identified as ddAMP, suggesting low levels of exonuclease contamination in the reaction mixture. All quantitative determinations were corrected for this background. A phosphatase-resistant compound (identified as [32P]ddAMP-terminated DNA primer/template was incubated with H9 cell extract and increasing amounts of unlabeled ATP or PP<sub>i</sub> as shown in Fig. 2. As expected, the addition of ATP to the reaction mixture resulted in increased formation of [32P]Ap<sub>4</sub>ddA (Fig. 2A and B), while the addition of PP<sub>i</sub> increased the formation of [32P]ddATP (Fig. 2C and D). In each case, the formation of the other removal products was diminished. The amounts of ATP and PP<sub>i</sub> present in the H9 cell extract were estimated from the linear portions of the plots of product versus added ATP or PP<sub>i</sub> as described in Methods. The results of multiple experiments are summarized in Table 1.

**Acceptor substrates for excision of ddAMP by HIV-1 RT are present in primary immune cell extracts.** Primary PBMCs were prepared from healthy donors, and purified cell subfractions were isolated by positive selection using magnetic beads conjugated with monoclonal antibodies to human CD4, CD8, and CD14 surface markers; the purity of each cell subfraction was greater than 96% (data not shown). Cell extracts were prepared from unstimulated or activated CD4<sup>T</sup> and CD8<sup>T</sup> T cells, 0.86 pmol; unstimulated CD4<sup>T</sup> and CD8<sup>T</sup> cells: 0.21 pmol; monocytes: 0.48 pmol/macrophages: 0.96 pmol/cell (51).

### Table 1. Intracellular levels of ATP and PP<sub>i</sub>

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Intracellular amt of:</th>
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<tr>
<td></td>
<td>ATP (pmol/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
</tr>
<tr>
<td>H9 cells</td>
<td>1,600 ± 300</td>
</tr>
<tr>
<td>Unstimulated CD4&lt;sup&gt;T&lt;/sup&gt; T cells</td>
<td>470 ± 60</td>
</tr>
<tr>
<td>Activated CD4&lt;sup&gt;T&lt;/sup&gt; T cells</td>
<td>1,230 ± 100</td>
</tr>
<tr>
<td>Unstimulated CD8&lt;sup&gt;T&lt;/sup&gt; T cells</td>
<td>360 ± 50</td>
</tr>
<tr>
<td>Activated CD8&lt;sup&gt;T&lt;/sup&gt; T cells</td>
<td>2,300 ± 340</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1,100 ± 230</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1,500 ± 390</td>
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<sup>a</sup> Reactions were performed as described in the legend for Fig. 2. Numbers are reported as ± SEM for two to five determinations. Data for primary cell subpopulations were obtained from five independent blood donors.

<sup>b</sup> Corrected for PP<sub>i</sub> formed during the extraction at 100°C as described in Materials and Methods. Corrections in pmol/10<sup>6</sup> cells: H9 cells, 7; unstimulated CD4<sup>T</sup> and CD8<sup>T</sup> T cells, 1.6; activated CD4<sup>T</sup> and CD8<sup>T</sup> T cells, 6.4; monocytes, 4; macrophages, 7. Correction for intracellular PP<sub>i</sub> concentration was 7.6 µM for all cell types.

<sup>c</sup> Concentrations calculated using the following values for cell volume: H9 cells, 1.2 pl/cell; unstimulated CD4<sup>T</sup> and CD8<sup>T</sup> T cells, 1.3; activated CD4<sup>T</sup> and CD8<sup>T</sup> T cells, 0.86 pl/cell; unstimulated CD4<sup>T</sup> and CD8<sup>T</sup> cells: 0.21 pl/cell; monocytes: 0.48 pl/cell; macrophages: 0.96 pl/cell (51).

**FIG. 1.** Excision of [32P]ddAMP by HIV-1 RT<sup>AZT</sup>, using acceptor substrates in H9 cell extract. (A) [32P]ddAMP-terminated L32 primer/WL50 template was incubated at 37°C without (−) or with (+) HIV-1 RT<sup>AZT</sup> and H9 cell extract for the times indicated at the bottom of the figure. The 40-min samples are shown before (lanes 2, 5, and 11) and after (lanes 3, 6, and 12) digestion with 2 units of calf intestinal phosphatase (CIP) for 15 min at 37°C. (B) Products expected from excision of [32P]ddAMP and transfer to various acceptor substrates.
almost exclusively (data not shown). This is most likely explained by the presence of a high concentration of PPi due to incomplete removal of platelets, which contain high levels of this acceptor substrate (20, 45, 49). The low levels of PPi detected in the extracts of unstimulated PBMC subpopulations suggest that platelets were effectively depleted during the purification of immune cell subfractions (Table 1).

When extracts from unstimulated CD4\(^{+}\) and CD8\(^{+}\) T cells provided the acceptor substrates for the removal reaction, the rate of formation of \([^{32}P]Ap_{4}dDA\) exceeded that of \([^{32}P]ddATP\) by threefold (Fig. 3A and C), indicating that ATP was the dominant substrate; however, when extracts prepared from activated cells were used in the removal reaction, the rate of formation of \([^{32}P]ddATP\) exceeded \([^{32}P]Ap_{4}dDA\) formation by eightfold for CD4\(^{+}\) T cells (Fig. 3B) and fivefold for CD8\(^{+}\) T cells (Fig. 3D), indicating that PPi was the dominant substrate in these extracts. Extracts from CD14\(^{+}\) monocytes formed \([^{32}P]Ap_{4}dDA\) and \([^{32}P]ddATP\) at similar rates (Fig. 3E), while extracts from differentiated macrophages supported the formation of \([^{32}P]Ap_{4}dDA\) at about two times the rate of \([^{32}P]ddATP\) formation (Fig. 3F).

Intracellular concentrations of ATP and PPi were estimated for each primary immune cell subpopulation as described for H9 cells (Table 1). Intracellular ATP concentrations ranged from 1.4 to 2.7 mM among different cell subpopulations and activation conditions, in agreement with published values (14, 19, 38, 53). Intracellular concentrations of PPi were estimated almost exclusively (data not shown). This is most likely explained by the presence of a high concentration of PPi due to incomplete removal of platelets, which contain high levels of this acceptor substrate (20, 45, 49). The low levels of PPi detected in the extracts of unstimulated PBMC subpopulations suggest that platelets were effectively depleted during the purification of immune cell subfractions (Table 1).

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![FIG. 2. Estimation of intracellular ATP and PPi concentrations in H9 cells. (A) \([^{32}P]ddAMP-terminated L32 primer/WL50 template was incubated with 3 \mu l H9 cell extract (3 \mu l corresponds to 3.4 \times 10^6 H9 cells) in the absence (−) or presence (+) of HIV-1 RT\(^{\text{AZT}}\) and additional ATP (concentrations given in \mu M at the bottom of the panel) in a total volume of 10 \mu l for 10 min at 37°C. The products were separated by electrophoresis as with Fig. 1. Each reaction condition is shown in duplicate except for the highest two ATP concentrations. (B) Rate of Ap\(_4\)dDA formation in the experiment in panel A as a function of added ATP concentration. Rates are given in nM Ap\(_4\)dDA formed after 10 min incubation. The inset shows the linear portion of the plot. Linear regression gave a slope (\(m\)) of 0.01 (nM increase in Ap\(_4\)dDA per \mu M increase in ATP) and y intercept (\(b\)) of 0.69 nM. The ratio (\(b/m = 69 \mu M\)) corresponds to the ATP concentration derived from the H9 cell extract (2,000 pmol ATP per 10^6 H9 cells). (C) Reactions were carried out as in (A) except that the 3-\mu l H9 cell extract added to the reaction mixture corresponded to 2.0 \times 10^5 H9 cells, and PPi was added instead of ATP (added PPi concentrations are indicated at the bottom of the panel). Phosphorimager exposure was longer than in (A) to increase sensitivity to the PPi excision product. All reactions are shown in duplicate except for the 0.8 to 6.4 \mu M PPi concentrations. (D) Rate of ddATP formation in the experiment shown in panel C as a function of added PPi concentration. Rates are given in nM ddATP formed after 10 min incubation. Slope (\(m = 1.13; nM increase in ddATP per \mu M increase in PPi\)) and y intercept (\(b = 0.31 nM\)) were determined by linear regression (inset). The ratio (\(b/m = 0.3 \mu M\)) corresponds to the PPi concentration derived from the H9 cell extract (15 pmol PPi per 10^6 H9 cells).]

![FIG. 3. Excision of ddAMP by HIV-1 RT\(^{\text{AZT}}\), using ATP and PPi in primary, human immune cell extracts. \([^{32}P]ddAMP-terminated L32 primer/WL50 template was incubated with HIV-1 RT\(^{\text{AZT}}\) and immune cell extract for various times at 37°C, and the products were identified by PAGE. Percentage of total radioactivity present in Ap\(_4\)dDA (closed circles) or ddATP (open circles) was determined by phosphorimaging. Reaction mixtures contained extracts from unstimulated CD4\(^{+}\) T cells (A), activated CD4\(^{+}\) T cells (B), unstimulated CD8\(^{+}\) T cells (C), activated CD8\(^{+}\) T cells (D), monocytes (E), or macrophages (F).]
to be 8 to 12 μM in unstimulated CD4+ and CD8+ T cells, increasing to 55 μM in activated CD8+ cells and 79 μM in activated CD4+ cells. The level of PPi, in CD14+ monocytes was 27 μM, which decreased to 7 μM upon differentiation into macrophages. Unstimulated CD4+ or CD8+ T cells and un-differentiated CD14+ monocytes were extracted immediately after the cell subpopulations were isolated, while stimulated T cells and differentiated macrophages were incubated in culture prior to extraction. It is possible that the concentrations of PPi and ATP were perturbed during the fractionation procedure. If so, this may affect the values obtained for the unstimulated cells. It is unlikely that any perturbation that occurred during cell fractionation would affect PPi or ATP concentrations after incubation in culture for at least 48 h. These results suggest that the levels of PPi are influenced by the activation status of the cells while ATP levels are much less affected.

Comparison of excision by RTWT and RTAZT in the presence of CD4+ T-cell extracts. The excision activities of RTWT and RTAZT were compared using different CD4+ T-cell extracts to determine conditions that provide the mutant enzyme with a greater ability to remove chain terminators. Figure 4A shows that the rate of excision of [32P]ddAMP from ddAMP-terminated primer/template by HIV-1 RTAZT was three- to sixfold greater than that by RTWT when the acceptor substrates were provided from an extract of unstimulated CD4+ T cells. This result is consistent with the demonstration that ATP is the dominant acceptor substrate in unstimulated CD4+ T cells (Fig. 3 and Table 1), since we and others have shown that primer unblocking by the mutant enzyme is greater than that of wild-type RT when the acceptor substrate is a nucleoside triphosphate (4, 5, 25, 28–30, 32, 42, 43). Similar results were observed for excision of [32P]AZTMP from AZTMP-terminated primer/template (Fig. 4B). When the acceptor substrates were provided by an extract of highly stimulated CD4+ T cells, no difference was observed in excision rates for RTWT and RTAZT (4, 5, 25, 28–30, 32, 43). This is consistent with the demonstration that PPi is the predominant acceptor substrate present in extracts of stimulated CD4+ T cells (Fig. 3 and Table 1) and that the rates of pyrophosphorolysis are similar for RTWT and RTAZT (4, 5, 25, 28–30, 32, 42, 43).

Excision by RTWT or RTAZT in the presence of mixtures of ATP and PPi. Since the preparation of cell extracts and addition of the reagents in the excision assay result in an overall dilution of the contents of the cell ranging from 30- to 100-fold, we compared the excision activities of RTWT and RTAZT in the presence of the major acceptor substrates, ATP and PPi, at concentrations that correspond to the estimated intracellular levels of these compounds. In a mixture containing 2.2 mM ATP and 10 μM PPi, the ATP-dependent removal reaction predominated (Fig. 5A and C). Comparison with the results for unstimulated CD4+ T-cell extract (Fig. 3A) indicates that the excision products are formed in approximately the same ratio. In a mixture containing 2.9 mM ATP and 80 μM PPi, the product ratio was reversed (Fig. 5B and D), in agreement with the results for activated CD4+ T cells (Fig. 3B). These results show that the relative contributions of ATP and PPi to the excision reaction are maintained upon extensive dilution.

To more specifically define the ATP/PPi conditions required for an excision advantage with the AZT-resistant enzyme, additional reactions were carried out with mixtures of 2.9 mM
ATP and concentrations of PPi ranging from 0.01 to 100 μM (Fig. 6). At low concentrations of PPi, total excision of ddAMP by RTAZT occurred at about 10 times the rate of excision by RTWT (Fig. 6A); however, this difference was smaller at PPi concentrations greater than 35 μM (for AZTMP removal). PPi-dependent excision predominated when the PPi concentration was less than 35 μM (for ddAMP removal). ATP-dependent excision predominated at low PPi concentrations but was suppressed when the PPi concentration exceeded about 10 μM (Fig. 6C). ATP-dependent excision predominated at low PPi concentrations but was suppressed when the PPi concentration was less than 10 μM (Fig. 6E).

Similar experiments were performed to measure the excision of AZTMP (Fig. 6B, D, and F). At low PPi concentrations, total excision of AZTMP by RTAZT occurred at about six times the rate of excision by RTWT; however, no difference was observed between RTWT and RTAZT at PPi concentrations greater than 50 μM (Fig. 6B). These results suggest that enhanced excision by RTAZT is only manifested in an environment where the PPi concentration is less than 35 μM (for ddAMP removal) or 50 μM (for AZTMP removal).

**DISCUSSION**

Cell extracts contain several compounds that can serve as acceptor substrates for HIV-1 RT-mediated excision. In most extracts tested, the predominant product was a dinucleoside tetraphosphate produced by ATP-mediated excision. The exceptions were activated primary human CD4+ and CD8+ T cells, where the predominant product was the deoxyoxynucleoside triphosphate produced by PPi-mediated excision. Our results suggest that concentrations of PPi in T lymphocytes are much lower than previously suggested (8 to 12 μM in unstimulated T cells and 55 to 79 μM in activated T cells); nonetheless, the PPi concentrations found in activated cells are sufficient to account for most of the excision, because the rate with PPi is much faster than with ATP. We tested mixtures of 2.9 mM ATP and various concentrations of PPi, and showed that ATP-dependent excision of ddAMP or AZTMP predominated when the PPi concentration was less than 35 μM or 50 μM, respectively. PPi-dependent excision predominated when PPi concentrations were greater than those values. These results present a paradox in terms of selection for AZT resistance mutations, since ATP-mediated excision, but not PPi-mediated excision, is elevated in RTAZT by comparison with RTWT (4, 5, 25, 28–30, 32, 42, 43). Yet our results have shown that the overall rate of excision is the same for RTAZT and RTWT when the reaction is carried out at the concentrations of ATP and PPi found in activated CD4+ T cells, where the bulk of viral replication and hence selection for AZT resistance mutations seems most likely to occur.

While there is evidence that cell-free virus particles are capable of reverse transcription, DNA synthesis occurs predominantly after entry of the viral core into the cytoplasm and appears to be coupled with disruption of the core structure (15). Biochemical analysis has identified a variety of nucleoprotein complexes that are active in DNA synthesis, although the significance of these complexes in the infection process has not been unambiguously demonstrated (10, 35). Structures active in reverse transcription have been identified cytologically by the ability to incorporate fluorescently labeled deoxynucleotides (26). The ability to label these cytoplasmic complexes with substrate indicates that they are permeable to deoxynucleotides and, presumably, also to ATP and PPi. The majority of reverse transcription complexes are attached to microtubules and cytoplasmic dynein, which are proposed to facilitate their movement to the perinuclear region in preparation for entry into the nucleus and integration in the host chromosomes (26).

The major site of cellular ATP synthesis in lymphocytes is the mitochondrial matrix, and ATP utilization occurs predominantly through protein synthesis, membrane transport, RNA and DNA synthesis, and maintenance of the mitochondrial proton gradient (6). Concentration gradients created by ATP consumption are reequilibrated through the action of enzymes such as adenylate kinase, which provides a relay system for rapid energy transfer from the mitochondria to the sites of energy utilization (9). Subcellular distribution of ATP was studied in four human cell lines, using luciferase constructs targeted to specific cellular compartments (12). Levels of ATP were similar in the cytosol, the subplasma membrane region, and the nucleus. A twofold-higher level was detected in the mitochondrial matrix. Therefore, the intracellular ATP concentration is maintained in a relatively narrow range (1 to 5 mM) in most cell types, including lymphocytes and lymphocyte-derived cell lines (8, 17, 53; this report), and it is likely that...
reverse transcription occurs in the presence of these concentrations.

Cellular PP<sub>i</sub> is formed in numerous metabolic reactions (44, 52), with major contributions from the activation of amino acids for protein synthesis, activation of fatty acids for oxidation, and formation of phosphodiester bonds in nucleic acid synthesis. It has been frequently suggested (21) that PP<sub>i</sub> is rapidly cleaved by inorganic pyrophosphatases located throughout the cell and that this lowers the local PP<sub>i</sub> concentration and energetically drives these reactions in the forward direction. Russell (44), however, has pointed out that the pyrophosphatase reaction does not attain equilibrium in vivo, at least in rat liver, and that PP<sub>i</sub> may have an additional role. It is possible that the PP<sub>i</sub> concentration may be elevated in certain circumstances, resulting in the transient reversal of PP<sub>i</sub>-generating reactions; however, in a general sense, biochemical or cellular mechanisms must exist that limit the extent of the reverse reactions to assure that biosynthetic reactions will go forward. The subcellular distribution of PP<sub>i</sub> and pyrophosphatases has not been extensively described; yet a wide range of PP<sub>i</sub> concentrations, ranging from 4 μM to 400 μM, has been reported in cell extracts (3, 8, 18, 24, 41, 44). This range is explained, at least in part, by events that occur during or after preparation of the cell extracts. We encountered several sources of variability. (i) Platelets, which contain very high levels of PP<sub>i</sub>, (8, 20, 45, 49), may be present at variable levels as a contaminant in unfractionated PBMC preparations. (ii) PP<sub>i</sub> may be lost during extraction due to residual enzyme activities that degrade PP<sub>i</sub> to inorganic phosphate or incorporate it into higher-molecular-weight compounds. (iii) PP<sub>i</sub> may be formed by the breakdown of other cellular constituents, such as phosphoribosyl pyrophosphate, during extraction (8).

De La Rosa et al. (8) have shown that specific procedures must be followed to obtain PBMC preparations that are free of platelets. These authors measured PP<sub>i</sub> at a level of 20 to 90 pmol/10<sup>6</sup> cells in unfractionated, unstimulated PBMC preparations (~30 to 210 μM, assuming an average cell volume of 0.42 pl for unfractionated PBMCs [8]). This value decreased to ~4 to 7 μM PP<sub>i</sub> when the platelet contamination was removed. The most commonly quoted value for PP<sub>i</sub> level is 133 ± 20 pmol/10<sup>6</sup> cells in unfractionated PBMCs, reported by Barshop et al. (3) (~130 to 400 μM, depending on assumptions about cell volume), but these authors did not address the possibility of platelet contamination or production of PP<sub>i</sub> from cellular constituents during perchloric acid extraction. High levels of PP<sub>i</sub> were also observed in unfractionated PBMCs in the present study (data not shown); however, PP<sub>i</sub> levels were much lower in PBMC subfractions, emphasizing the importance of platelet removal during isolation of cell subpopulations.

Cold methanol extraction, which yields satisfactory recovery of nucleotides (13, 37, 48), failed to inactivate enzymes that degrade [<sup>32</sup>P]PP<sub>i</sub>, or incorporate the label into higher-molecular-weight compounds during the extraction. Persistence of active enzymes after methanol extraction has been previously reported (36). We were able to minimize the loss of PP<sub>i</sub> during extraction by using boiling water as the extraction agent combined with MICROSEP filtration of the extracts. We also demonstrated that PP<sub>i</sub> is released by the breakdown of cellular constituents during the 100°C extraction procedure and that it was necessary to control the length of the heat treatment and to apply a correction for this background. We observed a 4- to 10-fold increase in PP<sub>i</sub> levels in CD8<sup>+</sup> and CD4<sup>+</sup> T cells after 48 h of mitogenic stimulation. This is larger than that previously reported after 8 h of stimulation (8); however, major metabolic changes that would lead to substantial PP<sub>i</sub> formation probably occur later than 8 h (39, 40). The level of PP<sub>i</sub> in primary CD14<sup>+</sup> monocytes (27 μM) decreased by about 75% after differentiation into macrophages, consistent with reduced proliferation in these terminally differentiated cells (27, 54).

There is general agreement that the rate of ATP-mediated excision is substantially greater for AZT-resistant RT than for WT RT (5, 16, 22, 25, 29, 32, 42, 43). While agreeing that ATP-mediated excision is the largest biochemical difference between mutant and WT RT and that an increase in PP<sub>i</sub>-mediated excision is not observed in kinetic assays, Ray et al. (42, 43) have suggested that pyrophosphorolysis may also play a role in AZT resistance. Mutant RT dissociates more slowly from AZTMP-terminating primer/template than WT RT (7), which could result in a net increase in formation of either PP<sub>i</sub> or ATP-mediated excision products. Ray et al. (42) have suggested that this difference may not be evident in kinetic assays. In addition, mutant RT is less sensitive than WT RT to inhibition by the next complementary deoxynucleoside triphosphate, which could lead to increased excision by the mutant enzyme using either PP<sub>i</sub> or ATP in the presence of physiological concentrations of deoxynucleoside triphosphates (43). Whether these effects are of sufficient magnitude to support a role for PP<sub>i</sub>-dependent excision in AZT resistance in vivo is yet to be determined.

A more general question is how selection of AZT-resistant mutants can occur when ATP-mediated excision may be overwhelmed by pyrophosphorolysis in actively replicating CD4<sup>+</sup> T cells. Assuming that reactions carried out with purified recombinant WT and mutant RTs and with synthetic chain-terminat ed primer/template are representative of the reactive species in the intracellular reverse transcription complexes, several possible explanations for this phenomenon should be considered: (i) The affinity of RT for PP<sub>i</sub> may be lower in vivo than that observed in vitro due to intracellular ionic conditions, the presence of cellular factors, etc., so that the ATP-dependent reaction may be sufficient to account for mutant selection in vivo. (ii) Alternative mechanisms for enhanced PP<sub>i</sub>-dependent excision by mutant enzyme not detected in vitro may oper ate in vivo as discussed above (42, 43). (iii) Access of PP<sub>i</sub> to the RT active site may be restricted by a mechanism that does not affect ATP-mediated excision. For example, a pyrophosphatase enzyme specifically bound to the reverse transcription complex, to cytoplasmic dynein, or to the microtubule network could prevent PP<sub>i</sub>-dependent excision during normal viral DNA synthesis and thereby prevent viral replication from inhibition by transient accumulation of PP<sub>i</sub>. (iv) PP<sub>i</sub> detected in whole-cell extracts may be primarily localized in vivo in a specific subcellular compartment where reverse transcription does not occur. (v) Selection for AZT resistance mutations may actually occur primarily in unstimulated or partially stimulated CD4<sup>+</sup> T cells, monocytes, or macrophages where the metabolite levels support the predominance of the ATP-dependent excision reaction. Currently available data do not allow us to determine whether any of these mechanisms plays a role in mutant selection.
In summary, our studies demonstrate that intracellular pools of ATP are sufficient to catalyze the excision of AZTMP or ddAMP from nascent DNA chains. Except for PPi in activated T cells, other substrates make only a minimal contribution to the total excision activity. A role for PPi in excision-mediated resistance cannot be excluded, but it seems more likely that mechanisms exist to ensure that DNA synthesis in the reverse transcription complex is not affected by fluctuations in the PPi concentration. The identification of factors that control excision in the intracellular environment will be important for our understanding of the in vivo selection of this class of drug resistance mutations.

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