1992

Phosphorylation of the Rex Protein of Human T-Cell Leukemia Virus Type 1

Yoshifumi Adachi
Kyoto University

Terry D. Copeland
National Cancer Institute

Chiaki Takahashi
Kyoto University

Tetsuya Nosaka
Kyoto University

Aftab Ahmed
Chapman University, aahmed@chapman.edu

See next page for additional authors

Recommended Citation

Adachi Y, Copeland TD, Takahshi C, et al. Phosphorylation of the Rex protein of human T-Cell leukemia virus type 1. *J. Biol. Chem.* 1992;267:21977-21981.

Phosphorylation of the Rex Protein of Human T-Cell Leukemia Virus Type 1

Authors
Yoshifumi Adachi, Terry D. Copeland, Chiaki Takahashi, Tetsuya Nosaka, Aftab Ahmed, Stephen Oroszlan, and Masakazu Hatanaka

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 30, Issue of October 25, pp. 21977-21981, 1992
Printed in U.S.A.

Phosphorylation of the Rex Protein of Human T-cell Leukemia Virus Type I*

(Received for publication, June 22, 1992)

Yoshifumi Adachi‡§, Terry D. Copeland§, Chiaki Takahashi‡, Tetsuya Nosaka‡, Aftab Ahmed§, Stephen Oroszlan§, and Masakazu Hatanaka‡¶

From the ‡Institute for Virus Research, Kyoto University, Kyoto 606, Japan and the §Laboratory of Molecular Virology and Carcinogenesis, Basic Research Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

Rex protein, the posttranscriptional regulator of human T-cell leukemia virus type I (HTLV-I), is required for the control of viral structural protein expression and virus replication. Rex is a phosphoprotein found predominantly in the cell nucleolus, whose function is thought to be regulated by its nucleolar localization and phosphorylation. Therefore, we investigated the *in vivo* phosphorylation of Rex protein in more detail. Phosphorylation of Rex occurred in all HTLV-I-infected cell lines examined *in vivo*, primarily at serine residues and to a very small extent at threonine residues. Treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) led to significant but transient enhancement of the incorporation of [^{32}P]orthophosphate into Rex protein. N-terminal truncation of Rex protein abolished TPA-dependent phosphorylation. Chymotryptic digestion of phosphorylated Rex yielded two phosphopeptides. *In vivo* phosphorylation sites were identified as serine residues 70 and 177 and threonine residue 174. Serine 70 was a TPA-dependent phosphorylation site within a regulatory domain. We have already shown that the protein kinase C inhibitor H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) specifically blocked accumulation of viral unspliced *gag-pol* mRNA. Therefore, the phosphorylation at serine 70 may be involved in the regulation of Rex function in response to extracellular stimuli.

Protein phosphorylation is believed to be a major general mechanism by which external physiological stimuli affect intracellular events in eukaryotic cells (1–3). Studies of the effect of phosphorylation on protein function have focused primarily on cell surface receptors and cytoplasmic proteins. However, it is clear that many nuclear proteins, including proteins thought to be involved in gene regulation, are also phosphorylated *in vivo* (1, 4–8). It therefore appears possible that the variable phosphorylation of these *trans*-acting regulatory proteins has functional consequences for the regulation of cellular or viral gene expression and may, in fact, constitute one mechanism by which nuclear events are modulated by external stimuli (1, 8).

HTLV-I[1] is an etiological agent for adult T-cell leukemia (9, 10). HTLV-I contains a unique regulatory gene, termed *pX*, that encodes three proteins, Tax, Rex, and pp21 (11–15). Tax is known to activate transcription of the virus (16–18) and several cellular genes (19–21). Rex is a posttranscriptional regulator that induces the accumulation of unspliced viral *gag-pol* mRNA (4, 5, 22, 23). Rex is a phosphoprotein found predominantly in the cell nucleolus, whose function may be regulated by its nucleolar localization (24) and phosphorylation (4, 5). Actually, dephosphorylation of Rex results in the decrease of accumulation of unspliced viral mRNA (4).

We were therefore interested in investigating in detail the *in vivo* phosphorylation of Rex protein. For this purpose, we isolated ^{32}P-labeled Rex from HTLV-I-infected human T-cell lines by immunoprecipitation, performed various biochemical analyses, and determined the phosphorylation sites within the Rex molecule.

EXPERIMENTAL PROCEDURES

Cells and Metabolic Labeling—HUT-102, MT-2, and TL-Su (25) are human T-cell lines infected with HTLV-I. TART-1 (26) is an HTLV-I-infected rat T-cell line. Cell lines CCRF-CEM, Jurkat, and Molt-4 (25) are uninfected human T-cell lines. Cell lines Raji and K-562 (25) are human lymphoblast-like cells and erythroleukemia cells, respectively. All cell lines were routinely maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (heat-inactivated at 56 °C for 30 min) at 37 °C in humidified air with 5% CO_2. COS-7 cells (5) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Metabolic labeling of cells with [^{32}P] orthophosphate (18.5 MBq/ml) was conducted by incubating cells (1×10^6 cells/ml) in phosphate-free medium supplemented with 10% fetal calf serum dialyzed against Tris-buffered saline (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5) at 37 °C for 2 h with various stimulations as previously described (27). At the end of the incubation period, cells were washed with phosphate-buffered saline (10 mM phosphate, 0.14 M NaCl, pH 7.5).

Antibody Preparation—Rabbits were immunized by injection of the synthetic peptide NH_2-Thr-Ser-Phe-Pro-Pro-Pro-Ser-Pro-Gly-Pro-Ser-Cys-Pro-Thr-COOH corresponding to the C-terminal region of HTLV-I Rex in complete Freund's adjuvant. An immunoglobulin G (IgG) fraction of antiserum was prepared as described previously (27) and used in this study.

Immunoblot Analysis—Proteins from each cell extract were subjected to SDS-PAGE on 12.5% polyacrylamide gels according to the procedure of Laemmli (28). Separated proteins were transferred onto nitrocellulose membrane and incubated with anti-Rex IgG. The im-

* This work was supported in part by grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, by grants-in-aid for scientific and cancer research from the Ministry of Education, Science and Culture of Japan, and by the National Cancer Institute, Department of Health and Human Services under Contract NO1-CO-74101. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; TLCK, 5-amino-L-tosylaminopentyl chloromethyl ketone; HPLC, high-pressure liquid chromatography; db-cAMP, N^2-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; db-cGMP, N^6-2′-*O*-dibutyrylguanosine 3′:5′-cyclic monophosphate; kb, kilobase(s); PVDF, polyvinylidene difluoride; HIV-1, human immunodeficiency virus type 1.

munocomplexes were detected by binding with horseradish peroxidase-conjugated protein A as described previously (25).

Immunoprecipitation—Metabolically labeled cells were lysed with RIPA buffer (50 mM Tris-HCl, 0.15 M NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM Na_3VO_3, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) at 4 °C for 30 min. After centrifugation at 15,000 rpm (Eppendorf microcentrifuge) for 10 min, the supernatant was incubated with anti-Rex IgG at 4 °C for 2 h. The immunocomplexes were precipitated with protein A-Sepharose CL-4B, then extensively washed with RIPA buffer and LSW buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.6) as described previously (27). The immunoprecipitates were subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, dried, and subjected to autoradiography using an intensifying screen.

Phosphoamino Acid Analysis—^{32}P-Labeled Rex protein was eluted from acrylamide gel sections by TPCK-trypsin digestion (50 μg/ml) in 50 mM NH_4HCO_3. Recovered phosphopeptides were hydrolyzed in 6 N HCl at 105 °C for 2 h. Resulting phosphoamino acid samples were mixed with standards (phosphoserine, phosphothreonine, and phosphotyrosine; 5 μg each) and applied onto cellulose-coated thin-layer glass plates (20 × 20 cm). High voltage electrophoresis was performed in pH 3.5 buffer (pyridine:acetic acid:water, 1:10:189 (v/v)) at 4 °C. ^{32}P-Labeled phosphoamino acids were identified by autoradiography, and phosphoamino acid standards were identified by spraying the plates with ninhydrin (27).

DNA Transfection—Rex expression vector pKCR27+40X was constructed by inserting a 1.1-kb *Bam*HI/*Sma*I fragment (nucleotides 5095–8308) of *pX* cDNA from pHA27X (24) into the same sites of pKCROHS (29). Deletion mutant pKCR21X was constructed by inserting a *Bal*I fragment (nucleotides 7437–8091) of λHTLVIC (30) into an *Hin*dIII site of pKCRH2 (31) by using *Hin*dIII linkers. COS-7 cells were transfected with plasmid DNA by the calcium phosphate-precipitation method (5).

Endoproteinase Digestion and HPLC Separation—TLCK-chymotrypsin was used for digestion of ^{32}P-labeled Rex on Immobilon PVDF membrane (Millipore). Digestions were carried out at 37 °C in 50 mM NH_4HCO_3 for 4 h followed by overnight incubation with a second identical dose of proteinase. The sample after digestion was subjected to reversed-phase HPLC using a C_{18} column (0.46 × 15 cm, Separation Group). The elution was performed by a linear gradient of 0–65% solvent B for 70 min (solvent A: 0.1% trifluoroacetic acid in water; solvent B: 0.1% trifluoroacetic acid in acetonitrile).

Radiosequencing—Each phosphopeptide from reversed-phase HPLC was subjected to sequential Edman degradation in a Beckman 890C automated spinning cup sequenator with 10 nmol of horse apomyoglobin (Sigma) carrier as described previously (32). Fractions collected at each cycle of sequential analysis were transferred to scintillation vials, dried with N_2 and heat, redissolved in 8 ml of Aquasure (Du Pont-New England Nuclear), and allowed to equilibrate in the dark for 2 h, and scintillation was counted.

RESULTS

In Vivo Phosphorylation of HTLV-I Rex—The Rex protein encoded by the HTLV-I *pX* gene open reading frame consists of 189 amino acids and has a molecular mass of 27 kDa. As shown in Fig. 1, all of the HTLV-I-infected cell lines that we tested (HUT-102, MT-2, TL-Su, and TART-1) expressed nearly the same amount of Rex protein. When the human hematopoietic cell lines were metabolically labeled with [^{32}P] orthophosphate for 2 h and subjected to immunoprecipitation, the radioactive Rex protein was specifically observed in the HTLV-I-infected cell lines (Fig. 2). In these cell lines, slightly limited proteolysis or microheterogeneity of labeled Rex was observed. The incorporation of $^{32}P_i$ into the Rex protein was enhanced approximately 5-fold when the cells were pulsed with 100 ng/ml TPA for 15 min at the end of ^{32}P-labeling period. Since the radioactivity associated with Rex was sensitive for the alkaline phosphatase treatment (data not shown), incorporation of the radioactivity was due to protein phosphorylation, but not ADP-ribosylation.

Effects of Drugs on Phosphorylation of Rex—To explore which protein kinases are involved in the phosphorylation of Rex *in vivo*, we examined the effects of various drugs on its phosphorylation. The phorbol ester TPA and the calcium

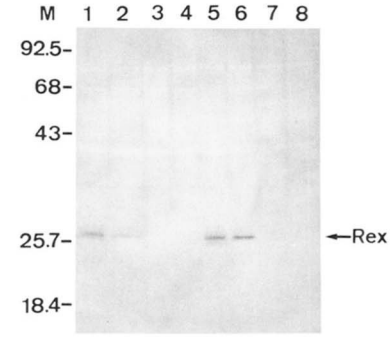

FIG. 1. **Detection of Rex in HTLV-I-infected human T-cells.** Cell extracts (120 μg of protein in every case) were analyzed by SDS-PAGE followed by immunoblotting with antibody to the C terminus of Rex as described under "Experimental Procedures." *Lane 1*, HUT-102; *lane 2*, MT-2; *lane 3*, Jurkat; *lane 4*, Molt-4; *lane 5*, TL-Su; *lane 6*, TART-1; *lane 7*, Raji; *lane 8*, K-562. Molecular size standards (in kilodaltons) are indicated on the left.

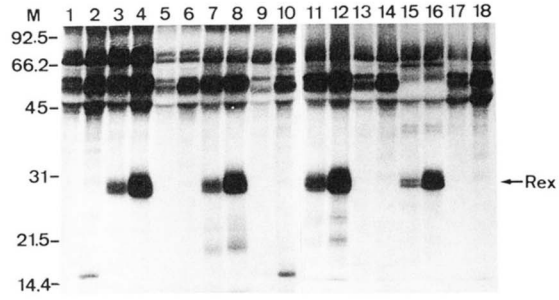

FIG. 2. ***In vivo* phosphorylation of Rex.** Cells were metabolically labeled with [^{32}P]orthophosphate, lysed, and subjected to immunoprecipitation with anti-Rex IgG as described under "Experimental Procedures." The cell lines examined were CCRF-CEM (*lanes 1* and *2*), HUT-102 (*lanes 3* and *4*), Molt-4 (*lanes 5* and *6*), MT-2 (*lanes 7* and *8*), Jurkat (*lanes 9* and *10*), TL-Su (*lanes 11* and *12*), Raji (*lanes 13* and *14*), TART-1 (*lanes 15* and *16*), and K-562 (*lanes 17* and *18*). Samples in *even-numbered lanes* were pulsed with 100 ng/ml TPA for 15 min after 2 h of labeling. Samples in *odd-numbered lanes* were not subjected to TPA treatment. Autoradiography was carried out for 24 h. Molecular size standards (in kilodaltons) are indicated on the left.

ionophore A_{23187} are known to activate cellular protein kinase C (1, 33). Forskolin and db-cAMP are activators of adenylate cyclase and protein kinase A (27), respectively. Protein kinase G is known to be activated by db-cGMP (27). The HTLV-I-infected cell lines TL-Su and HUT-102 were pulsed with 100 ng/ml TPA, 10 μM forskolin, 0.5 mM db-cAMP, or 0.5 mM db-cGMP for 15 min at the end of the ^{32}P-labeling period, and ^{32}P-labeled Rex was immunoprecipitated. As shown in Fig. 3, the incorporation of $^{32}P_i$ into Rex was preferentially and rapidly augmented by TPA-treatment but not by treatment with any other drugs. By contrast, no increased synthesis of Rex protein was observed during 15-min drug treatment (data not shown). Quantification of the resultant autoradiograms by densitometric scanning revealed an approximate 5-fold increase of Rex phosphorylation in the TPA-treated cultures relative to the control or to other treatments.

Effect of Down-regulation of Protein Kinase C on Rex Phosphorylation—Stimulation of cells with TPA appears to result in rapid translocation of protein kinase C from the cytosol to sites within the membrane. However, this activated membrane-associated protein kinase C is rapidly degraded by nonlysosomal proteolysis, leading to a subsequent rapid decline in kinase activity (down-regulation) (34, 35). In order to confirm the possible involvement of protein kinase C on Rex phosphorylation, we caused the down-regulation of cellular protein kinase C by long-term exposure to TPA and moni-

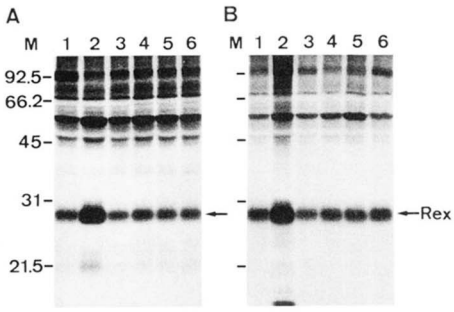

FIG. 3. **Effect of drugs on phosphorylation of Rex.** Cells were labeled with [^{32}P]orthophosphate for 2 h, then incubated with various drugs at optimum concentrations for 15 min as described under "Experimental Procedures." Cell extracts were immunoprecipitated with anti-Rex IgG and analyzed by SDS-PAGE followed by autoradiography. The cell lines examined were TL-Su (*A*) and HUT-102 (*B*). *Lane 1*, no stimulation; *lane 2*, 100 ng/ml TPA; *lane 3*, 10 μM A_{23187}; *lane 4*, 10 μM forskolin; *lane 5*, 0.5 mM db-cAMP; *lane 6*, 0.5 mM db-cGMP. Molecular size standards (in kilodalton) are indicated on the left.

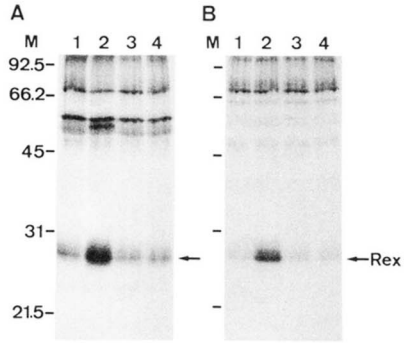

FIG. 4. **Effect of down-regulation of protein kinase C on phosphorylation of Rex.** Cell lines HUT-102 (*A*) and TL-Su (*B*) were preincubated overnight in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of 200 ng/ml TPA, then incubated with [^{32}P] orthophosphate for 2 h as described under "Experimental Procedures." In *lanes 2* and *4*, samples were incubated with 100 ng/ml TPA for 15 min after the labeling period. In *lanes 1* and *3*, samples were labeled without TPA treatment. Cell extracts were immunoprecipitated with anti-Rex IgG and analyzed by SDS-PAGE followed by autoradiography. Molecular size standards (in kilodaltons) are indicated on the left.

tored the phosphorylation of Rex after retreatment with TPA. TPA-dependent enhancement of Rex phosphorylation was observed in the absence of pretreatment with TPA (Fig. 4*A*, *lanes 1* and *2*). By contrast, the phosphorylation of Rex was no longer dependent on TPA after extensive pretreatment with TPA (*lanes 3* and *4*). However, TPA-independent and basal phosphorylation of Rex, which corresponds to the control, was still detectable. Similar results were also observed in another HTLV-I-infected cell lines, TL-Su (Fig. 4*B*). Thus, a part of Rex phosphorylation was closely related to the activation of protein kinase C.

Phosphoamino Acid Analysis of Rex—To identify the phosphorylated amino acids present in Rex, ^{32}P-labeled Rex proteins from various HTLV-I-infected cells and drug-treated cells were eluted from gel sections and subjected to acid hydrolysis. Phosphoamino acids were separated by electrophoresis and detected by autoradiography. As shown in Fig. 5, in all HTLV-I-infected cells (*A*) and all drug treatments (*B*), the Rex protein was phosphorylated mainly at the serine residue with trace amounts of phosphothreonine *in vivo*. However, phosphotyrosine was not detectable in any of the cells and treatments examined.

Phosphorylation of Truncated Rex, pp21—The objectives of

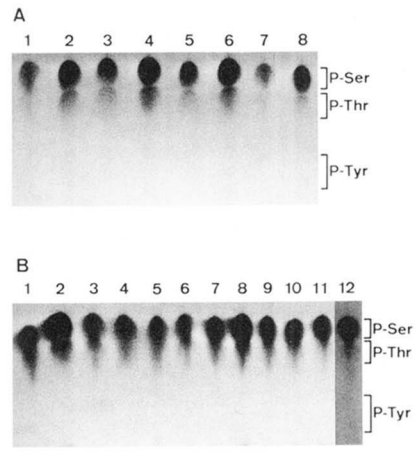

FIG. 5. **Phosphoamino acid analysis of Rex.** *A*, ^{32}P-labeled Rex protein from various HTLV-I-infected human T-cells was immunoprecipitated, eluted from gels, and hydrolyzed in 6 N HCl at 105 °C for 2 h. Phosphoamino acids were separated by high voltage electrophoresis and subjected bo autoradiography. Rex proteins were isolated from MT-2 (*lanes 1* and *2*), HUT-102 (*lanes 3* and *4*), TL-Su (*lanes 5* and *6*), and TART-1 (*lanes 7* and *8*). Samples in *lanes 2*, *4*, *6*, and *8* were treated with TPA for 15 min after the labeling period. Samples in *lanes 1*, *3*, *5*, and *7* were labeled without TPA treatment. *B*, ^{32}P-labeled Rex proteins from drug-treated HUT-102 (*lanes 1–6*) and TL-Su (*lanes 7–12*) were subjected to phosphoamino acid analysis as described above. *Lanes 1* and *7*, no treatment; *lanes 2* and *8*, 100 ng/ml TPA; *lanes 3* and *9*, 10 μM A_{23187}; *lanes 4* and *10*, 10 μM forskolin; *Lanes 5* and *11*, 0.5 mM db-cAMP; *lanes 6* and *12*, 0.5 mM db-cGMP.

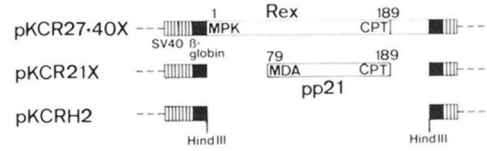

FIG. 6. **Schematic presentation of Rex expression plasmid and deletion mutant.** pKCR27+40X in which *pX* cDNA was inserted downstream of the simian virus 40 early promoter, encodes full-size Rex. The exon and intron sequences of the rabbit β-globin gene are indicated by *solid boxes*. pKCR21X, which produces pp21, was constructed by deleting the first 78 amino acids of Rex. pKCRH2 is the original vector used for construction of the above plasmids.

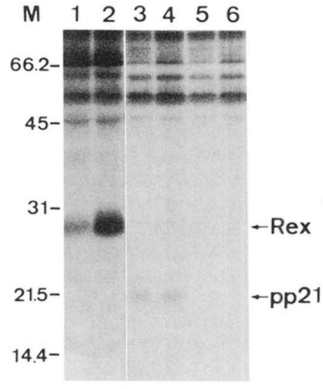

FIG. 7. **Phosphorylation of Rex and pp21 in transfected COS-7 cells.** The products of Rex expression vector (pKCR27+40X: *lanes 1* and *2*), deletion mutant (pKCR21X: *lanes 3* and *4*), and negative control (pKCRH2: *lanes 5* and *6*) were immunoprecipitated from ^{32}P-labeled cell lysates after parallel DNA-transfection. Samples in *lanes 2*, *4*, and *6* were incubated with TPA for 15 min after 2 h of labeling. Samples in *lanes 1*, *3*, and *5* were labeled without TPA treatment. Molecular size standards (in kilodaltons) are indicated on the left.

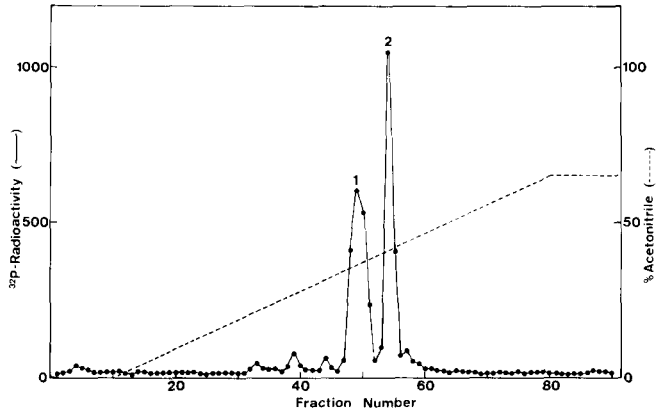

FIG. 8. **Purification of chymotryptic phosphopeptides by C_{18} reversed-phase HPLC.** ^{32}P-Labeled Rex was prepared by immunoprecipitation and SDS-PAGE, then digested with TLCK-chymotrypsin as described under "Experimental Procedures." The resulting peptides were applied onto a C_{18} column (0.46 × 15 cm). The phosphopeptides were separated at a flow rate of 1.0 ml/min with a linear gradient of 0–65% acetonitrile in 0.1% trifluoroacetic acid as shown by the *dashed line*. The radioactivity in each fraction was monitored by Cerenkov counting.

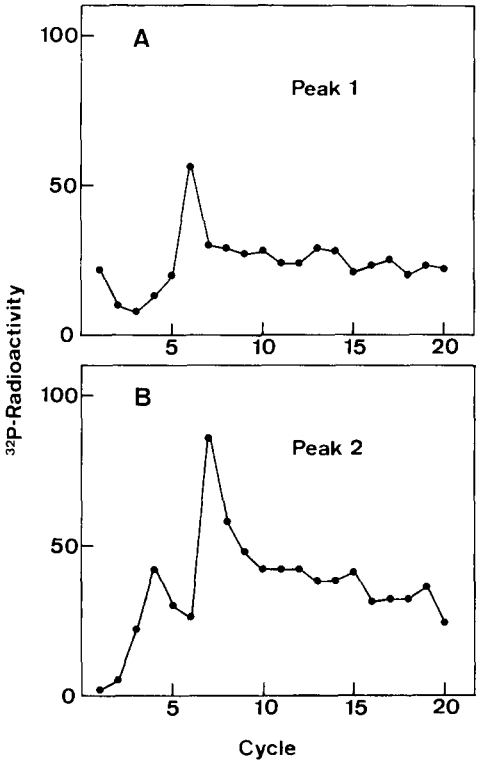

FIG. 9. **Radiosequence analysis.** Each chymotryptic phosphopeptide from reversed-phase HPLC was subjected to automated Edman degradation as described under "Experimental Procedures." The amounts of ^{32}P radioactivity released during each cycle are shown. *A*, *peak 1* in Fig. 8; *B*, *peak 2* in the same figure.

this study were to explore the possibility of phosphorylation in a highly basic N-terminal sequence, MPKTRRRPRRSQRKRPPTPWP, which is the nucleolar targeting signal of Rex (24), and to estimate the TPA-dependent phosphorylation site(s), roughly. For this purpose, we constructed the expression vectors pKCR27+40X (for full-size Rex) and pKCR21X (for pp21, by deletion of the 78 N-terminal amino acid residues of Rex) (Fig. 6). After DNA transfection of these expression vectors into COS-7 cells, the cells were metabolically labeled with $^{32}P_i$ in the absence or presence of TPA and analyzed by

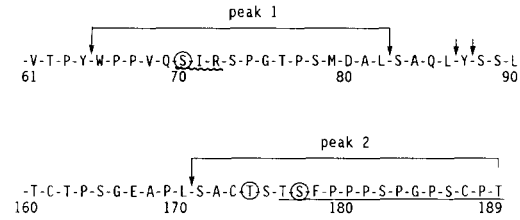

FIG. 10. ***In vivo* phosphorylation sites of HTLV-I Rex.** *Arrows* mark the sites of efficient chymotryptic cleavage. *Brackets* indicate chymotryptic phosphopeptides, identified by the numbering system of Fig. 8. *Circled* residues are phosphorylated *in vivo*. The consensus sequence of the site phosphorylated by protein kinase C is marked with a *wavy underline*. The antigenic sequence of the antibody used in this study is *underlined*.

immunoprecipitation followed by SDS-PAGE and autoradiography. As shown in Fig. 7, the Rex protein expressed by pKCR27+40X was phosphorylated *in vivo*, and TPA-dependent phosphorylation was also observed in COS-7 cells. The deletion of the first 78 amino acid residues of Rex caused a significant disappearance of TPA-dependent phosphorylation. However, phosphorylation of pp21, which corresponds to the basal level of TPA-independent phosphorylation was still observed. Hence, TPA-dependent site(s) are located in the 78 N-terminal amino acid residues, and another site(s) is located in residues 79–189 of Rex.

Identification of in Vivo Phosphorylation Sites of Rex—In order to determine the precise location of the phosphorylated serine(s) and threonine(s), we prepared ^{32}P-labeled Rex by metabolic labeling followed by immunoprecipitation. After extensive digestion with TLCK-chymotrypsin, the resulting peptides were separated by HPLC (Fig. 8). Two ^{32}P-labeled phosphopeptides were isolated. Since the phosphopeptide in peak 2 was immunoreactive with antibody against a synthetic peptide that represented the 14 C-terminal amino acid residues of Rex (data not shown), it was identified as the C-terminal chymotryptic peptide, SACTSTSFPPPSPGPSCPT (residues 171–189). This and the results obtained with the N-terminally truncated Rex (Fig. 7) permitted us to postulate that peak 1 should be the chymotryptic peptide containing TPA-dependent phosphorylation site(s) within the 78 N-terminal residues. Each ^{32}P-labeled chymotryptic phosphopeptide was mixed with myoglobin as carrier protein and subjected to automated Edman degradation in a spinning cup sequenator. Successive degradation and the sequence cycle number were monitored by analysis of phenylthiohydantoin amino acids of carrier protein released at cycles 2 and 11. The amount of radioactivity released during each cycle was monitored by scintillation counting to determine the position(s) of phosphorylated amino acids relative to the amino end of the peptides (Fig. 9). ^{32}P radioactivity was released during the sixth cycle of peak 1 (Fig. 9*A*). Only one predicted chymotryptic peptide (residues 65–82) contains a serine in this position. Moreover, Ser-70 coincides with the consensus sequence of phosphorylation sites of protein kinase C (Fig. 10). A similar analysis was used to identify the phosphorylation site(s) of the C-terminal chymotryptic peptide (peak 2 in Fig. 8). In this case, ^{32}P radioactivity was released at the fourth and seventh cycles (Fig. 9*B*). These positions of the chymotryptic peptide (residues 171–189) (Fig. 10) corresponded to Thr-174 and Ser-177, respectively.

DISCUSSION

In this study, we examined the *in vivo* phosphorylation of the HTLV-I posttranscriptional regulator, Rex. Phosphorylation of Rex was observed in all human T-cell lines bearing HTLV-I proviruses (HUT-102, MT-2, TL-Su, and TART-1;

Fig. 2) tested and in COS-7 cells transfected by the Rex expression vector (Fig. 7, *lanes 1* and *2*). In every case, the major phosphoamino acids of phosphorylated Rex were phosphoserine with a trace amount of phosphothreonine. Tyrosine kinase was not involved, since phosphotyrosine was not detected in any of the HTLV-I-infected cells with or without drug treatment. The level of Rex phosphorylation was enhanced by the treatment of cells with TPA alone, a specific activator of cellular protein kinase C (1, 33). Moreover, the pretreatment of cells with TPA caused down-regulation of cellular protein kinase C, resulting in no augmentation of TPA-dependent Rex phosphorylation after retreatment with TPA. These results closely mirror the previously reported effect of TPA on protein kinase C activation in cultured cells (1, 33–36).

The *in vivo* phosphorylation sites of Rex were identified as Ser-70, Ser-177, and Thr-174 by radiosequencing. As the deletion of the first 78 amino acid residues of Rex caused a significant disappearance of TPA-dependent phosphorylation, Ser-70 was identified as a TPA-dependent phosphorylation site. This is supported by inspection of its surrounding sequences. Ser-70 fits an apparent consensus sequence of the phosphorylation site by protein kinase C, Ser*/Thr*-*X*-Arg/Lys (37). A wide range of *in vivo* protein kinase C substrate proteins has been reported. However, very few of these proteins are localized predominantly in the nuclei, especially in the nucleolus (1, 38). Indeed, it has been reported that only low levels of protein kinase C activity are associated with the nuclei (1, 39). Therefore, TPA-dependent phosphorylation of Rex may (i) be directly mediated by protein kinase C in the cytoplasm and the phosphorylated Rex is translocated into the nucleolus, or (ii) reflect an additional step of signal transduction, resulting in activation of a nuclear kinase, such as the human immunodeficiency virus type 1 (HIV-1) Rev kinase (39).

Recent studies have revealed that (i) the first 77 N-terminal amino acids of Rex constitutes a domain with RNA-binding activity (40), (ii) a region between residues 55 and 132 is required for Rex-mediated *trans* activation (41), and (iii) an effector domain (residues 66–118) is essential for Rex function (42). In this study, we identified that the TPA-dependent phosphorylation site, Ser-70 is located within these functional regions. Protein phosphorylation has been recognized as a major posttranslational regulatory mechanism and is thought to play an important role in the control of cell growth, differentiation, and tumorigenicity (1, 2). Hence, it is possible that phosphorylation at Ser-70 is necessary for the activation of Rex function. We have demonstrated that the protein kinase inhibitor H-7 blocks accumulation of viral unspliced *gag-pol* mRNA corresponding with the decreased *in vivo* phosphorylation of Rex (4). It has been reported that TPA is an inducer of viral gene expression and replication of HTLV-I or HIV-1 in infected human T-cells (43, 44) and enhances Rev phosphorylation *in vivo* (45). Thus, retroviruses such as HTLV-I and HIV-1 that encode their own *trans*-regulator proteins would be able to adapt if they could also respond to regulatory signals by extracellular stimuli. One way by which this effect could be accomplished would be having the function of Rex be dependent on phosphorylation.

Acknowledgments—We thank Drs. Deborah Morrison and Tetsu Akiyama for helpful suggestions. We also thank Anne Arthur for editorial assistance and Cheri Rhoderick for preparation of the manuscript.

REFERENCES

1. Nishizuka, Y. (1986) *Science* **233,** 305–312
2. Hunter, T., and Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54,** 897–930
3. Cohen, P. (1982) *Nature* **296,** 613–620
4. Adachi, Y., Nosaka, T., and Hatanaka, M. (1990) *Biochem. Biophys. Res. Commun.* **169,** 469–475
5. Nosaka, T., Siomi, H., Adachi, Y., Ishibashi, M., Kubota, S., Maki, M., and Hatanaka, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9798–9802
6. Curran, T., Miller, A. D., Zokas, L., and Verma, I. M. (1984) *Cell* **36,** 259–268
7. Patskan, G. J., and Baxter, C. S. (1985) *J. Biol. Chem.* **260,** 12899–12903
8. Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., and Montminy, M. R. (1988) *Nature* **334,** 494–498
9. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 7415–7419
10. Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S., and Miyoshi, I. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6476–6480
11. Seiki, M., Hattori, S., Hirayama, Y., and Yoshida, M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3618–3622
12. Lee, T. H., Coligan, J. E., Sodroski, J. G., Haseltine, W. A., Salahuddin, S. Z., Wong-Staal, F., Gallo, R. C., and Essex, M. (1984) *Science* **226,** 57–61
13. Slamon, D. J., Shimotohno, K., Cline, M. J., Golde, D. W., and Chen, I. S. Y. (1984) *Science* **226,** 61–65
14. Kiyokawa, T., Seiki, M., Iwashita, S., Imagawa, K., Shimizu, F., and Yoshida, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8359–8363
15. Nagashima, K., Yoshida, M., and Seiki, M. (1986) *J. Virol.* **60,** 394–399
16. Sodroski, J., Rosen, C., Goh, W. C., and Haseltine, W. (1985) *Science* **228,** 1430–1434
17. Chen, I. S. Y., Slamon, D. J., Rosenblatt, J. D., Shah, N. P., Quan, S. G., and Wachsman, W. (1985) *Science* **229,** 54–58
18. Fujisawa, J., Seiki, M., Kiyokawa, T., and Yoshida, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 2277–2281
19. Inoue, J., Seiki, M., Taniguchi, T., Tsuru, S., and Yoshida, M. (1986) *EMBO J.* **5,** 2883–2888
20. Fujii, M., Sassone-Corsi, P., and Verma, I. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8526–8530
21. Miyatake, S., Seiki, M., Yoshida, M., and Arai, K. (1988) *Mol. Cell. Biol.* **8,** 5581–5587
22. Inoue, J., Yoshida, M., and Seiki, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3653–3657
23. Hidaka, M., Inoue, J., Yoshida, M., and Seiki, M. (1988) *EMBO J.* **7,** 519–523
24. Siomi, H., Shida, H., Nam, S. H., Nosaka, T., Maki, M., and Hatanaka, M. (1988) *Cell* **55,** 197–209
25. Adachi, Y., Takano, E., Murachi, T., and Hatanaka, M. (1988) *Biol. Chem. Hoppe-Seyler* **369,** 223–227
26. Tateno, M., Kondo, N., Itoh, T., Chubachi, T., Togashi, T., and Yoshiki, T. (1984) *J. Exp. Med.* **159,** 1105–1116
27. Adachi, Y., Ishida-Takahashi, A., Takahashi, C., Takano, E., Murachi, T., and Hatanaka, M. (1991) *J. Biol. Chem.* **266,** 3968–3972
28. Laemmli, U. K. (1970) *Nature* **227,** 680–685
29. Sabe, H., Tanaka, A., Siomi, H., Koyasu, S., Yonehara, S., Yahara, I., Tagaya, Y., Sugie, K., Teshigawara, K., Yodoi, J., and Hatanaka, M. (1988) *Int. J. Cancer* **41,** 880–885
30. Mori, K., Sabe, H., Siomi, H., Iino, T., Tanaka, A., Takeuchi, K., Hirayoshi, K., and Hatanaka, M. (1987) *J. Gen. Virol.* **68,** 499–506
31. Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M., and Numa, S. (1984) *Nature* **307,** 604–608
32. Shenoy, S., Choi, J.-K., Bagrodia, S., Copeland, T. D., Maller, J. L., and Shalloway, D. (1989) *Cell* **57,** 763–774
33. Pasti, G., Lacal, J. C., Warren, B. S., Aaronson, S. A., and Blumberg, P. M. (1986) *Nature* **324,** 375–377
34. Chida, K., Kato, N., and Kuroki, T. (1986) *J. Biol. Chem.* **261,** 13013–13018
35. Ase, K., Berry, N., Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1988) *FEBS Lett.* **236,** 396–400
36. Adachi, Y., Maki, M., Ishii, K., Hatanaka, M., and Murachi, T. (1990) in *The Biology and Medicine of Signal Transduction* (Nishizuka, Y., ed) pp. 478–484, Raven Press, New York
37. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985) *J. Biol. Chem.* **260,** 12492–12499
38. Chan, P. K., Aldrich, M., Cook, R. G., and Busch, H. (1986) *J. Biol. Chem.* **261,** 1868–1872
39. Cochrane, A., Kramer, R., Ruben, S., Levine, J., and Rosen, C. A. (1989) *Virology* **171,** 264–266
40. Grassmann, R., Berchtold, S., Aepinus, C., Ballaun, C., Boehnlein, E., and Fleckenstein, B. (1991) *J. Virol.* **65,** 3721–3727
41. Hofer, L., Weichselbraun, I., Quick, S., Farrington, G. K., Böhnlein, E., and Hauber, J. (1991) *J. Virol.* **65,** 3379–3383
42. Hope, T. J., Bond, B. L., McDonald, D., Klein, N. P., and Parslow, T. G. (1991) *J. Virol.* **65,** 6001–6007
43. Harada, S., Koyanagi, Y., Nakashima, H., Kobayashi, N., and Yamamoto, N. (1986) *Virology* **154,** 249–258
44. Siekevitz, M., Josephs, S. F., Dukovich, M., Peffer, N., Wong-Staal, F., and Greene, W. C. (1987) *Science* **238,** 1575–1578
45. Hauber, J., Bouvier, M., Malim, M. H., and Cullen, B. R. (1988) *J. Virol.* **62,** 4801–4804