Site-Specific Phosphorylation of the Human Immunodeficiency Virus Type-1 Rev Protein Accelerates Formation of an Efficient RNA-Binding Conformation†

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ABSTRACT: Phosphorylation is important in the regulation of many cellular processes, yet the precise role of protein phosphorylation for many RNA-binding protein substrates remains obscure. In this report, we demonstrate that phosphorylation of a recombinant human immunodeficiency virus type-1 Rev protein promotes rapid formation of an efficient RNA-binding state. The apparent dissociation constant for ligand binding is enhanced 7-fold for the protein following phosphorylation; however, phosphate addition leads to a 1.6-fold decrease in RNA ligand−protein complex stability. RNA ligand binding stimulates slow formation of an equally competent binding state for the unphosphorylated protein, indicating that the addition of phosphate or ligand binding promotes a similar conformational change in Rev. Phosphorylation directly alters the conformation of Rev, as revealed by modification experiments that monitor the solvent accessibility of cysteines in the protein. These biochemical properties are attributed to the addition of phosphate at one of two serine residues (Ser-54 or Ser-56) that lie within the multimerization domain adjacent to the RNA-binding helix. Glutaraldehyde-mediated cross-linking experiments revealed that phosphorylation of Rev does not affect Rev multimerization activity. The Rev protein from the less pathogenic HIV-2 isolate lacks this phosphorylation site in the amino acid sequence; thus, the described biochemical properties of the phosphorylated protein may contribute to Rev activity and possibly to HIV-1 virulence during natural infection.

Protein modification by the reversible addition of phosphate represents a universal mechanism for post-translational regulation of biological activity. Protein phosphorylation serves an important role in the control of gene expression and signaling developmental changes (Musti et al., 1997; Watanabe et al., 1997). Numerous examples of both positive and negative regulation of nucleic acid-binding proteins by protein phosphorylation have been documented. The specific DNA-binding activity of the G-box binding factor 1 (GBF1)[1] from *Arabidopsis* is positively stimulated following phosphorylation and may lead to changes in GBF1-responsive gene expression (Klimczak et al., 1992). Phosphorylation of the c-Jun proto-oncogene product increases its transactivation potential and DNA-binding activity due to a reduction in c-Jun ubiquination and consequent stabilization of the protein (Musti et al., 1997). The RNA-binding activity of the HTLV-I and the HTLV-II viral Rex post-transcriptional activator protein for its RNA ligand is markedly increased following phosphorylation *in vivo* (Green et al., 1992). Protein phosphorylation of other RNA-binding proteins has a negative impact on RNA-binding functions. A 28 kDa RNA-binding protein from spinach chloroplasts exhibits reduced binding to its RNA ligand when phosphorylated, suggesting that phosphorylation may affect the role of this protein in 3′-end processing or translation of the target RNA ligands (Lisitsky & Schuster, 1995). The phosphorylation of the 60 kDa component of the *psbA* mRNA-binding protein complex from *Chlamydomonas reinhardtii* reduces the RNA-binding activity of the protein complex and may account for the reduced translation of *psbA* mRNA upon transfer of cells to dark growth conditions (Danon & Mayfield, 1994). While phosphorylation is an important regulator of function for a variety of nucleic acid-binding proteins, the precise role of protein phosphorylation for many RNA-binding protein substrates remains obscure.

The Rev protein from the human immunodeficiency virus type I, similar to the HTLV-I/II Rex protein, functions as an essential post-transcriptional regulator of virion gene expression (Sodroski et al., 1986). Rev mediates transactivation by inhibiting spliceosome formation and by enhancing nucleocytoplasmic transport of viral transcripts containing its ligand RNA-binding site, the Rev responsive element (RRE) (Feinberg et al., 1986; Kjems et al., 1991; Malim et al., 1989b). Rev exists as a phosphoprotein *in vivo* during viral infection or transient expression of the gene. The biochemical properties of HIV-1 Rev were initially determined *in vitro* with the use of recombinant proteins that are synthesized in an unphosphorylated form in bacteria (Zapp & Green, 1989; Daly et al., 1989; Cochrane et al., 1989a). Phosphorylation of Rev occurs exclusively on serine residues (Hauber et al., 1988; Cochrane et al., 1989b,c) and is modulated by phorbol esters (Hauber et al., 1988), suggesting that a serine/threonine protein kinase component

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[1] Abbreviations: GBF1, G-box binding factor 1; HTLV-I and -II, T-cell leukemia viruses types I and II; HIV-1, human immunodeficiency virus type-1; RRE, Rev responsive element; PCR, polymerase chain reaction; HMK, heart muscle kinase; PKC, protein kinase C.

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of a signal transduction pathway is responsible for this modification *in vivo*. Here we present a detailed characterization of one novel role that protein phosphorylation has upon the biochemical properties of Rev, the accelerated formation of an efficient RNA-binding conformation for the protein.

MATERIALS AND METHODS

Materials. All buffer solutions were prepared with sterile water that was initially deionized using a Millipore MilliQ Plus water purification system. All buffer components that were used in this work were of the highest grade obtainable from various manufacturers. Bacteriophage T7 RNA polymerase was purified following overexpression of the bacteriophage T7 *gene 1* from BL21[pAR1219] (Davanloo et al., 1984). Calf intestinal alkaline phosphatase was obtained from United States Biochemicals; all other enzymes were obtained from New England Biolabs. The [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from DuPont−New England Nuclear.

Construction of Recombinant Protein Mutant Genes. The protein mutants that contain H6Rev[S54A] and H6Rev-[S56A] were generated using oligonucleotide primers that encode the desired codon changes and PCR (Mullis & Faloona, 1987). The H6Rev genes containing the mutations were reconstructed using standard recombinant DNA techniques (Ausubel et al., 1992) and verified by cycle sequencing procedures (Sears et al., 1992).

Preparation of RNA. The synthetic RRE RNA was transcribed from an *Eco*RI linearized pGEM4RRE DNA template using T7 RNA polymerase (Celander & Nussbaum, 1996). The stem−loop IIB RNA was prepared in a similar fashion from a partially double-stranded oligonucleotide transcription template (Milligan & Uhlenbeck, 1989). The RRE and stem−loop IIB RNA transcripts were gel-purified and labeled as described previously (Celander & Nussbaum, 1996). The stem−loop IIB RNA molecule was dephosphorylated by calf intestinal alkaline phosphatase and 5′-^{32}P end-labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (6000 Ci/mmol). The radiolabeled RNA product was repurified by electrophoresis on a denaturing gel before use in the RNA−protein binding assays.

Preparation of Recombinant Rev Proteins. The recombinant Rev protein and mutants were prepared in the following manner. The *E. coli* strain SG22094 (MC4100/ ΔclpP::CM Δlon rcsA::Δkan; kindly provided by S. Gottesman) was transformed to ampicillin resistance with a derivative of pCWH6Rev (kindly provided by A. Cochrane). A 1 L culture of SG22094[pCWH6Rev] was grown to an OD_{600} = 0.40, and recombinant protein expression was induced by the addition of IPTG to a final concentration of 1 mM. The recombinant protein was purified from crude lysates by Ni-NTA chromatography (Qiagen) according to the manufacturer's instructions. The protein preparation was dialyzed against storage buffer [50 mM sodium citrate (pH 6.5), 150 mM NaCl, 20% glycerol, and 0.1 mM PMSF].

Phosphorylation Assays. The HIV-1 H6Rev protein (2.4 μM) was phosphorylated in 1 × kinase buffer [20 mM Tris-HCl (pH 7.5), 1 mM DTT, 100 mM NaCl, 12 mM $MgCl_2$, and 200 μM ATP (0.748 Ci/mmol of [γ-^{32}P]ATP)] containing either protein kinase C (0.05 unit) or HMK (50 units). The H6Rev protein and kinase were preincubated together at 30 °C for 10 min before addition of ATP and $MgCl_2$. The extent of phosphorylation was monitored over time by quenching aliquots of reaction mixtures with an excess of EDTA, followed by electrophoresis of samples on a 15% SDS−PAGE gel system. The standard phosphorylation reaction conditions were used for the phosphorylation protection experiments that are presented in Figure 2 except that H6Rev protein (2.4 μM) was preincubated with either RRE RNA or stem−loop IIB RNA (2.4 μM) in 1 × kinase buffer at 20 °C for 1 min before HMK (5 units) was added. Gels were washed briefly with 50% methanol following electrophoresis and dried. The radioactivity in the dried gels was analyzed using a Molecular Dynamics PhosphorImager. The phosphate content in each radiolabeled protein band was calculated from ^{32}P-labeled specific activity standards that were exposed on the same phosphor screen as the dried SDS−PAGE gels.

Chromatographic Analysis of ^{32}P-Labeled Phosphopeptides Generated by PKC and HMK. The H6Rev protein was phosphorylated *in vitro* using standard phosphorylation reaction conditions except that 4 μg of H6Rev and 1.7 μM [γ-^{32}P]ATP (6000 Ci/mmol) were incubated with 50 units of HMK or 0.05 unit of PKC at 30 °C for 3 h. The phosphoproteins were subjected to partial digestion with TPCK-treated trypsin in 50 mM NH_4HCO_3 (pH 8.0). The peptides were TCA-precipitated, and the pellets were resuspended in 100 μL of 0.1% trifluoroacetic acid in water. Chromatography was performed on a Waters Spherisorb S5 ODS2 C18 analytical HPLC column (4.6 × 250 mm) equipped with a C18 guard column. An aliquot of the resuspended peptides (10 μL) was loaded onto the column equilibrated in 94% buffer A (0.1% trifluoroacetic acid in water), 6% buffer B (0.1% trifluoroacetic acid in acetonitrile), and the column was washed at a flow rate of 1 mL/min. The peptides were eluted from the column with a linear gradient to 100% buffer B over 60 min at a flow rate of 1 mL/min. Radioactive fractions were detected using an online Beckman Model 171 radioisotope detector.

Phosphopeptide Analyses. Phosphorylated H6Rev protein was prepared using the standard phosphorylation reaction conditions except that unlabeled ATP was substituted for [γ-^{32}P]ATP and the reaction was performed at 30 °C for 3 h. Mock reactions were prepared in a reaction mixture lacking ATP and $MgCl_2$. Unphosphorylated and phosphorylated Rev protein samples (∼850 pmol each) were incubated with TPCK-treated trypsin (20 μg) in 50 mM NH_4HCO_3 (pH 8.0) (50 μL) for 13−17 h at 37 °C. The tryptic digests were subjected to reverse phase HPLC-ESIMS using a microbore C18 reverse phase column (Vydac) with a gradient of acetonitrile (5−65%) in 0.1% trifluoroacetic acid at a flow rate of 40 μL/min. The ESIMS spectra were assigned on a Micromass (Manchester, U.K.) Quattro I triple quadrupole mass spectrometer in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois at Urbana−Champaign.

RNA−Protein Binding Experiments. Unphosphorylated and phosphorylated H6Rev proteins were prepared as described in *Phosphopeptide Analyses*. Serial dilutions of the reaction mixtures were prepared in cold (2 °C) TK buffer [43 mM Tris-HCl (pH 8.0), 50 mM KCl] containing 30 μg/mL BSA. Protein-excess RNA-binding experiments were performed in TK buffer containing 5 mM DTT, 0.45 μg of yeast $tRNA^{Phe}$, and 50 μg/mL BSA. A constant concentration of 5′-^{32}P-labeled stem−loop IIB RNA (<100 pM) was

mixed with varying concentrations of phosphorylated or unphosphorylated H6Rev protein in a reaction volume of 500 μL. The reactions were filtered through BA85 Protran nitrocellulose filters following incubation on ice for a period of 20 min to 5 h. The amount of radioactivity retained on the filter was determined by a Beckman liquid scintillation counter. The data were fit to a retention efficiency and a K_d value assuming a bimolecular equilibrium for each experiment. The rate of complex dissociation was determined using the filter-binding method following the addition of 1000-fold excess of cold stem−loop IIB RNA to the binding mixtures containing preformed RNA−protein complexes (20 nM).

Sulfhydryl Group Modification Experiments. Phosphorylated H6Rev was prepared in reactions containing H6Rev (7.9 μM), HMK (1.67 units/μL), 200 μM ATP (0.748 Ci/mmol of [γ-^{32}P]ATP), and 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, and 12 mM $MgCl_2$. Mock reactions were incubated in the kinase buffers lacking ATP and $MgCl_2$. Following incubation at 30 °C for 3 h, the protein mixtures were dialyzed against TK buffer (pH 8.0). Protein recovery was determined by phosphorimaging methods for ^{32}P-labeled H6Rev and by silver staining procedures for unlabeled H6Rev using known ^{32}P-labeled specific activity standards and protein standards, respectively. H6Rev or pH6Rev (0.6 μM) was incubated with fluorescein-5-maleimide (77 μM) in TK buffer pH 8.0 at 22 °C. The optimum concentration of maleimide compound for use in these studies was within the linear range of the reagent's reactivity, as assessed by independent experiments (not shown). Aliquots were removed from each modification reaction over time and quenched with L-cysteine at a final concentration of 143 mM. The reaction products were resolved by SDS−PAGE. Immunoblots were prepared by electrophoretic transfer of proteins from SDS−PAGE gels onto nitrocellulose membranes. Membrane-bound fluoresceinated proteins were detected with a rabbit anti-fluorescyl IgG polyclonal antibody and a horseradish peroxidase linked goat anti-rabbit IgG (Zymed) and visualized with a Renaissance Western blot chemiluminescence reagent (DuPont NEN; Ausubel et al., 1992). The extent of modification was determined by laser densitometry. Data reported represent an average of two independent experiments for each modification reaction.

Glutaraldehyde-Mediated Protein Cross-Linking Assays. Phosphorylated H6Rev was prepared in reactions containing H6Rev (7.9 μM), HMK (1.67 units/μL), 200 μM ATP (0.748 Ci/mmol of [γ-^{32}P]ATP), and 20 mM HEPES−NaOH (pH 7.5), 100 mM NaCl, 1 mM DTT, and 12 mM $MgCl_2$. Mock-phosphorylated H6Rev was incubated in the kinase buffers lacking ATP. Following incubation at 30 °C for 3 h, the proteins were mixed with glutaraldehyde to a final concentration of 0.025% and incubated at 22 °C. Aliquots were removed over time, quenched in 167 mM glycine (pH 7.0), and subjected to SDS−PAGE. Samples were visualized by either autoradiography or silver staining procedures.

RESULTS AND DISCUSSION

Rev was phosphorylated by the catalytic subunits of two representative serine/threonine kinases, protein kinase C and the cAMP-dependent protein kinase from bovine heart muscle (Figure 1a). Mass spectral analyses of H6Rev and the phosphorylated species (pH6Rev) revealed an 80.2 amu

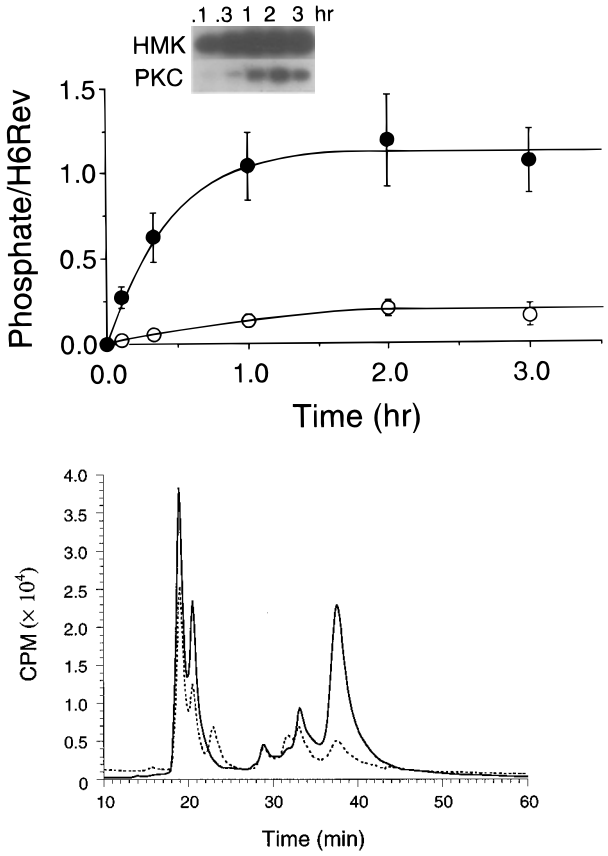

FIGURE 1: Phosphorylation of H6Rev by either protein kinase C or heart muscle kinase. (a) Phosphorylation of H6Rev carried out by the catalytic subunit of either protein kinase C (○) or heart muscle kinase (●). (b) HPLC profiles of phosphopeptides generated by partial digestion with trypsin following phosphorylation of H6Rev by protein kinase C (---) or heart muscle kinase (—). Unincorporated ^{32}P from the labeling reaction eluted from the column between 0 and 10 min.

increase in the parent molecular ion peak for the phosphorylated protein, which is compatible with the addition of a single phosphate moiety (not shown). Phosphorylation occurs exclusively on serine as assessed by a phosphoamino acid analysis of pH6Rev (not shown). Previous studies demonstrated that protein kinase C serves as one of the phosphorylating kinases of the Rev protein *in vivo* (Hauber et al., 1988). We were unable to phosphorylate H6Rev with protein kinase C to yields comparable to that observed with HMK, presumably owing to the low active concentrations of the commercially available enzyme. Protein kinase C and HMK recognize and phosphorylate identical sites within many substrates (Pearson & Kemp, 1991); therefore, we examined whether the same site of H6Rev was phosphorylated *in vitro* by both. H6Rev was phosphorylated with [γ-^{32}P]ATP using each of the kinases, and the resultant pH6Rev samples were subjected to a partial digest using trypsin. The phosphopeptide mixtures were analyzed by HPLC. The retention profiles of all ^{32}P-labeled phosphopeptide products generated with HMK were also found in samples prepared with protein kinase C (Figure 1b). These data indicate that a predominant site phosphorylated by both kinases is the same.

Three experiments were performed to determine the location of the phosphorylated serine residue in the protein. The Rev protein binds specifically to a high-affinity binding site (termed stem−loop IIB) in the Rev responsive element

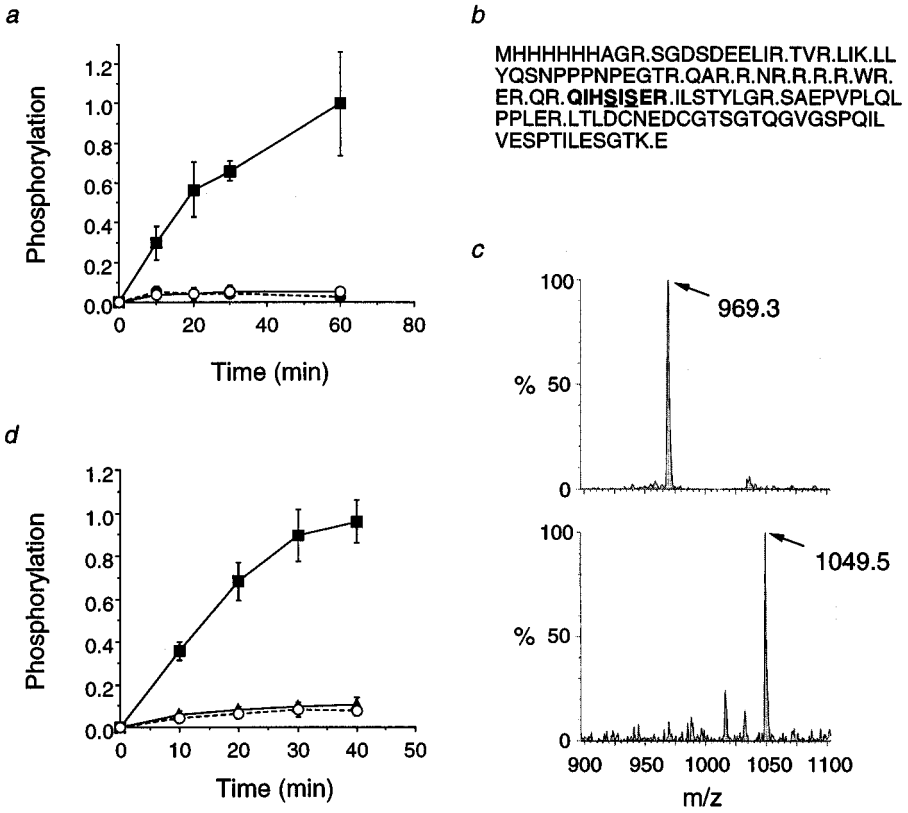

FIGURE 2: Characterization of the phosphorylation site in H6Rev. (a) Phosphorylation of H6Rev (2.4 μM) in association either with no RNA ligand (■) or with an equimolar amount of either RRE RNA (○) or stem−loop IIB RNA (●). (b) Predicted pattern of products for the H6Rev protein following a complete tryptic digestion is denoted by periods. The boldface peptide encodes residues 51−58 of Rev and contains the phosphorylation site in underlined serine residues. (c) Electrospray mass spectrometry of chromatographic fractions for tryptic fragments generated from unphosphorylated H6Rev (upper panel) and phosphorylated H6Rev (lower panel). The major peak present in each panel (denoted as 100%) represents the molecular ion peak differences for tryptic digest patterns generated for unphosphorylated and phosphorylated protein species following deconvolution of the mass spectral data. (d) Rate of phosphorylation for unsubstituted H6Rev (■), H6Rev[S54A] (○), and H6Rev[S56A] (▲).

(RRE) RNA with a dissociation constant of ~3 nM (Iwai et al., 1992; Heaphy et al., 1990). Phosphorylation experiments with H6Rev−RNA complexes were conducted to evaluate whether the serine substrate remains accessible to phosphorylation. The rate of phosphorylation of H6Rev is markedly reduced for protein−RNA complexes that contain either RRE RNA or stem−loop IIB RNA as compared with reaction mixtures that lack RNA (Figure 2a). These RNA molecules do not influence the rate or the extent of phosphorylation of casein (not shown). Peptide analyses of H6Rev and pH6Rev were performed to map the site of phosphorylation. H6Rev and pH6Rev were digested with trypsin, and the resulting tryptic fragments were subjected to LC/MS analyses using a VG Quattro mass spectrometer fitted with an electrospray (ESI) ion source. The peak fractions contained multiple molecular mass peaks. The molecular mass data found in each chromatographic fraction corresponded to specific tryptic peptides generated for both H6Rev and pH6Rev with the exception of one peptide (Figure 2b). This peptide (QIHSISER; 969.3 amu) is abundant in the H6Rev tryptic digest but is reduced to near background levels in the corresponding chromatographic fraction from the pH6Rev tryptic digest (Figure 2c). The pH6Rev tryptic digest contains a new peptide with a molecular mass of 1049.5 amu that is not evident in the tryptic digest of H6Rev. We interpret the increase of 80.2 amu to correspond to one phosphate group attached to the tryptic fragment QIHSISER of pH6Rev. The phosphorylation site in H6Rev lies in proximity to the helix that contains the RNA-binding domain in the native structure. The two serine residues in this region were individually changed to alanine using site-directed mutagenesis to determine which one of the serines was the phosphoacceptor site. The resulting H6Rev[S54A] and H6Rev[S56A] protein mutants were expressed in *E. coli* strain SG22094, purified by Ni-NTA chromatography, and subjected to phosphorylation *in vitro*. The protein mutants were poorly phosphorylated under the same reaction conditions that resulted in efficient phosphorylation of the unsubstituted H6Rev protein (Figure 2d). Extended substrate recognition sequences occur frequently for protein kinases (Zetterqvist et al., 1990) and are apparent in this case. We cannot discriminate the serine that represents the phosphoacceptor amino acid from the serine that comprises part of an extended phosphorylation substrate sequence within this peptide.

The phosphorylation site resides next to the RNA-binding helix of Rev (Tan et al., 1993); therefore, we investigated whether phosphorylation of the protein alters its RNA-binding characteristics. Filter-binding experiments with excess protein were performed using stem−loop IIB RNA with either H6Rev or pH6Rev (Figure 3a). The H6Rev−RNA complex displays a K_d of ~2 nM, which is comparable to dissociation constant data obtained with other recombinant Rev proteins with similar RNA ligands (Iwai et al., 1992; Heaphy et al., 1990). The pH6Rev−RNA complex displays a K_d of ~0.3 nM. These data suggest that phosphorylation enables H6Rev to bind the RNA ligand 7-fold more tightly than unphosphorylated H6Rev. A kinetic profile of H6Rev−

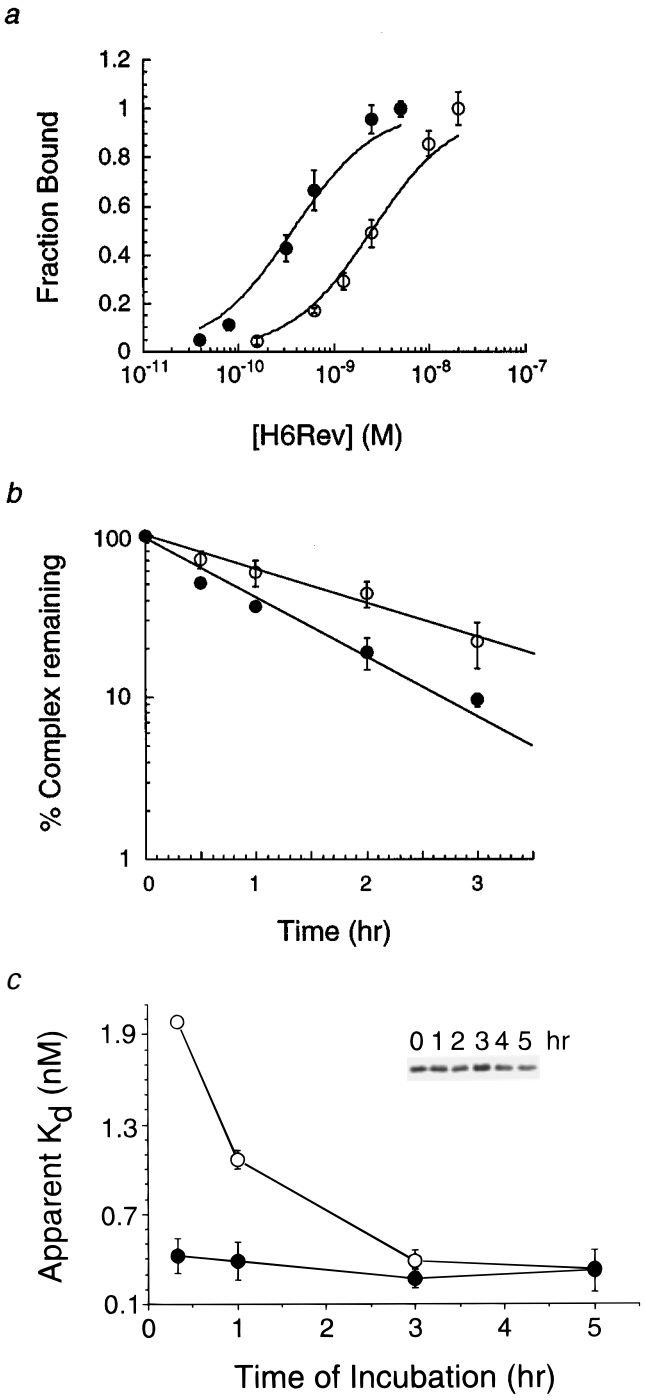

FIGURE 3: Phosphorylation accelerates formation of an efficient RNA-binding state. (a) RNA−protein binding equilibrium plots for complexes formed; (b) rate of dissociation of preformed complexes; (c) effect of apparent dissociation constant for complex formation as a function of time. In (a−c), complexes contain stem−loop IIB RNA formed with either unphosphorylated H6Rev (○) or phosphorylated H6Rev (●). The inset in (c) shows the stability of H6Rev protein over the course of the incubation period. The reactions were filtered through BA85 Protran nitrocellulose filters following incubation on ice for 20 min (a) or longer (c). The data shown in (a, c) reflect an average of at least three independent experiments. The data shown in (b) reflect an average of at least two independent experiments.

RNA complex dissociation was performed to evaluate whether phosphorylation stabilizes the resultant protein−RNA complexes. Protein−RNA complexes that contain pH6Rev dissociate ~1.6-fold more rapidly than observed for the corresponding complexes that contain H6Rev (Figure 3b); thus, phosphorylation destabilizes the pH6Rev−RNA complexes. The apparent dissociation constants for pH6Rev−RNA complexes remain unchanged over time, but the apparent K_d values for the H6Rev−RNA complexes decrease over time and approach the corresponding values for the pH6Rev−RNA complexes (Figure 3c).

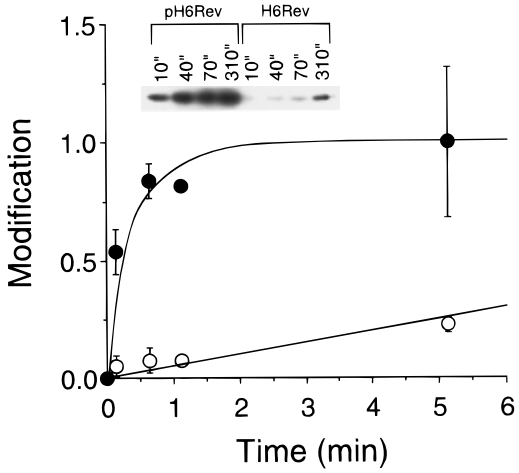

FIGURE 4: Phosphorylation affects H6Rev protein conformation. Sulfhydryl group accessibility in pH6Rev (●) or H6Rev (○) was monitored by fluorescein-5-maleimide modification. Inset: Immunoblot results of kinetic assay for maleimide modification of pH6Rev and H6Rev as detected with an anti-fluorescyl IgG polyclonal antibody.

These results suggest that phosphorylation of Rev leads to the formation of an efficient RNA-binding conformation. A chemical modification assay was used to investigate whether surfaces of the protein are differentially accessible to solvent following protein phosphorylation. H6Rev and pH6Rev were incubated with a fluoresceinated maleimide derivative that specifically reacts with surface-accessible sulfhydryl groups. Modification reactions were quenched with excess L-cysteine and the products were fractionated by SDS−PAGE. Figure 4 illustrates a relative kinetic index of protein modification as obtained by immunoblot analyses of fluoresceinated protein samples using a rabbit anti-fluorescyl IgG polyclonal antibody (Watt et al., 1980). The rate of cysteine modification is ~20-fold greater for pH6Rev than that observed for H6Rev. Phosphorylation of H6Rev promotes a conformational change in the protein that results in greater accessibility of one or more cysteines (C-85 or C-89) to the solvent.

Rev activity is dependent upon its ability to form higher order multimeric species (Zapp et al., 1991; Malim & Cullen, 1991). The site of protein phosphorylation physically coincides with the multimerization domain (Zapp et al., 1991), and we examined whether phosphorylation affected the ability of H6Rev to undergo multimerization. Protein samples were incubated with glutaraldehyde to promote formation of cross-linked species, and aliquots of the reaction mixture were quenched in glycine and analyzed by SDS−PAGE. The cross-linked products generated with H6Rev and pH6Rev were visualized by silver staining or autoradiography. The extent of cross-linked multimers was virtually indistinguishable between phosphorylated and unphosphorylated Rev (Figure 5). Similar experiments were performed with the H6Rev[S54A] and H6Rev[S56A] protein mutants. The multimerization properties of these mutants resemble those described for unsubstituted H6Rev (Zapp et al., 1991; Figure 5), indicating that the alanine substitutions do not

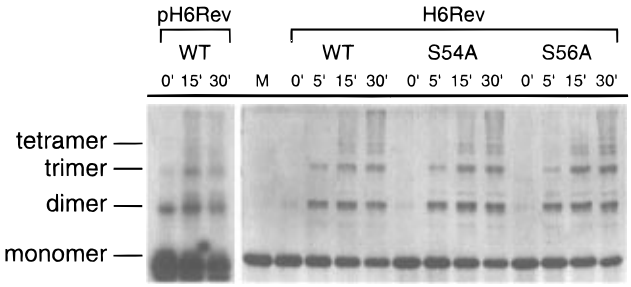

FIGURE 5: Phosphorylation does not alter the multimerization activity of H6Rev. Glutaraldehyde-mediated cross-linking assays were used to evaluate multimerization properties of pH6Rev, H6Rev, H6Rev[S54A], and H6Rev[S56A] as a function of time. The locations of monomers and multimers in the gel are indicated. An un-cross-linked H6Rev sample is shown in lane M.

significantly disrupt the structure of the multimerization domain.

The biochemical studies described in this report reveal new insights about one role of protein phosphorylation in HIV-1 Rev function. Phosphorylation induces conformational changes in the protein that accompany efficient RNA-binding activity; however, the unphosphorylated protein appears to achieve, albeit more slowly, an equivalent RNA-binding state. Phosphorylation of Ser-54/Ser-56 or RNA ligand binding may facilitate rapid folding of Rev's conformation necessary for the protein's full biological activity. The impact of Ser-54/Ser-56 phosphorylation on Rev protein conformation would be realized under circumstances where Rev−RRE RNA complex formation is unfavored due to limiting amounts of RRE RNA-containing transcripts expressed during natural infection. Rev binding to the RRE RNA promotes the assembly of additional Rev molecules on the RNA for activity (Malim & Cullen, 1991) under conditions where both Rev protein and RRE RNA-containing transcripts are abundantly expressed during productive infection. The observation that RNA ligand binding to Rev blocks specific phosphate addition to Ser-54/Ser-56 suggests that site-specific phosphorylation of Ser-54/Ser-56 may not be required for Rev activity when RRE RNA-containing transcripts are abundant. Previous studies that investigated the linkage between protein phosphorylation and functional activity for Rev *in vivo* therefore may not have been sensitive enough to detect the biochemical properties identified in this study (Cochrane et al., 1989b; Malim et al., 1989a). Phosphorylation of additional serines in Rev may regulate other functions of the protein such as the ability of Rev to form multimers (Olsen et al., 1990; Malim & Cullen, 1991; Zapp et al., 1991) or to interact with specific cellular factors of the transport apparatus (Fankhauser et al., 1991; Ruhl et al., 1993; Luo et al., 1994; Bogerd et al., 1995; Stutz et al., 1995; Fritz et al., 1995).

How general is phosphorylation as a regulator of Rev-like activity for other human retroviruses? The HTLV Rex protein, which performs similar functions in HTLV as the Rev protein displays in HIV, is also phosphorylated *in vivo* and appears to possess enhanced RNA-binding activity *in vitro* (Green et al., 1992). It remains to be established whether phosphorylation of Rex accelerates the formation of an efficient RNA-binding conformation for the protein as described here for HIV-1 Rev. One prospective clinical study found that HIV-1- and HIV-2-associated AIDS incidence rates are distinct, and the disease-free survival period for HIV-2 is significantly longer when compared with HIV-1 (Marlink et al., 1994). The two related HIV viruses therefore display different biological properties that undoubtedly reflect a combination of factors, including potential differences in virus replication efficiency and virus load during natural infection. Phosphorylation cannot affect the conformation of the HIV-2 Rev protein in the same manner as described here for the HIV-1 Rev protein because HIV-2 Rev contains alanine substitutions for the serines located in the corresponding region of that protein (Madore et al., 1994). One might anticipate a strong selective pressure to maintain this region in the HIV-1 Rev protein for this function, as well as provide opportune targets for the development of antiviral strategies.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds. (1992) in *Short Protocols in Molecular Biology*, 2nd ed., John Wiley & Sons, New York.

Bogerd, H. P., Fridell, R. A., Madore, S., & Cullen, B. R. (1995) *Cell 82*, 485−494.

Celander, D. W., & Nussbaum, J. M. (1996) *Biochemistry 35*, 12061−12069.

Cochrane, A. W., Chen, C.-H., Kramer, R., Tomchak, L., & Rosen, C. A. (1989a) *Virology 173*, 335−337.

Cochrane, A. W., Golub, E., Volsky, D., Ruben, S., & Rosen, C. A. (1989b) *J. Virol. 63*, 4438−4440.

Cochrane, A., Kramer, R., Ruben, S., Levine, J., & Rosen, C. A. (1989c) *Virology 171*, 264−266.

Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E., & Rusche, J. R. (1989) *Nature 342*, 816−819.

Danon, A., & Mayfield, S. P. (1994) *EMBO J. 13*, 2227−2235.

Davanloo, P., Rosenberg, A. H., Dunn, J. J., & Studier, W. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2035−2039.

Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P., & Laemmli, U. K. (1991) *Mol. Cell. Biol. 11*, 2567−2575.

Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C., & Wong-Staal, F. (1986) *Cell 46*, 807−817.

Fritz, C. C., Zapp, M. L., & Green, M. R. (1995) *Nature 376*, 530−533.

Green, P. L., Yip, M. T., Xie, Y., & Chen, I. S. Y. (1992) *J. Virol. 66*, 4325−4330.

Hauber, J., Bouvier, M., Malim, M. H., & Cullen, B. R. (1988) *J. Virol. 62*, 4801−4804.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Karn, J., Lowe, A. D., Singh, M., & Skinner, M. A. (1990) *Cell 60*, 685−693.

Iwai, S., Pritchard, C., Mann, D. A., Karn, J., & Gait, M. J. (1992) *Nucleic Acids Res. 20*, 6465−6472.

Kjems, J., Frankel, A. D., & Sharp, P. A. (1991) *Cell 67*, 169−178.

Klimczak, L. J., Schindler, U., & Cashmore, A. R. (1992) *Plant Cell 4*, 87−98.

Lisitsky, I., & Schuster, G. (1995) *Nucleic Acids Res. 23*, 2506−2511.

Luo, Y. L., Yu, H., & Peterlin, B. M. (1994) *J. Virol. 68*, 3850−3856.

Madore, S. J., Tiley, L. S., Malim, M. H., & Cullen, B. R. (1994) *Virology 202*, 186−194.

Malim, M. H., & Cullen, B. R. (1991) *Cell 65*, 241−248.

Malim, M. H., Böhnlein, S., Hauber, J., & Cullen, B. R. (1989a) *Cell 58*, 205−214.

Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., & Cullen, B. R. (1989b) *Nature 338*, 254−257.

Marlink, R., Kanki, P., Thior, I., Travers, K., Eisen, G., Siby, T., Traore, I., Hsieh, C. C., Dia, M. C., Gueye, E. H., Hellinger, J., Guèye-Ndiaye, A., Sankalé, J. L., Ndoye, I., Mboup, S., & Essex, M. (1994) *Science 265*, 1587−1590.

Milligan, J. F., & Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51−62.

Mullis, K. B., & Faloona, F. A. (1987) *Methods Enzymol. 155*, 335−350.

Musti, A. M., Treier, M., & Bohmann, D. (1997) *Science 275*, 400−402.

Olsen, H. S., Cochrane, A. W., Dillon, P. J., Nalin, C. M., & Rosen, C. A. (1990) *Genes Dev. 4*, 1357−1364.

Pearson, R. B., & Kemp, B. E. (1991) *Methods Enzymol. 200*, 62−81.

Ruhl, M., Himmelspach, M., Bahr, G. M., Hammerschmid, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G. K., Probst, H., Bevec, D., & Hauber, J. (1993) *J. Cell Biol. 123*, 1309−1320.

Sears, L. E., Moran, L. S., Kissinger, C., Creasey, T., Perry-O'Keefe, H., Roskey, M., Sutherland, E., & Slatko, B. E. (1992) *Biotechniques 13*, 626−633.

Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E., & Haseltine, W. A. (1986) *Nature 321*, 412−417.

Stutz, F., Neville, M., & Rosbash, M. (1995) *Cell 82*, 495−506.

Tan, R., Chen, L., Buettner, J. A., Hudson, D., & Frankel, A. D. (1993) *Cell 73*, 1031−1040.

Watanabe, Y., Shinozaki-Yabana, S., Chikashige, Y., Hiraoka, Y., & Yamamoto, M. (1997) *Nature 386*, 187−190.

Watt, R. M., Herron, J. N., & Voss, E. W., Jr. (1980) *Mol. Immunol. 17*, 1237−1243.

Zapp, M. L., & Green, M. R. (1989) *Nature 342*, 714−716.

Zapp, M. L., Hope, T. J., Parslow, T. G., & Green, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7734−7738.

Zetterqvist, Ö., Ragnarsson, U., & Engström, L. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed. pp 171−187, CRC Press, Boca Raton, FL.

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