Mass Spectrometry of UV-Cross-Linked Protein–Nucleic Acid Complexes: Identification of Amino Acid Residues in the Single-Stranded DNA-Binding Domain of Human Replication Protein A

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Photochemical cross-linking of human replication protein A (hRPA) to oligonucleotide dT₃₀ was performed to enable identification of amino acid sequences that reside in the DNA-binding domain. A nucleoprotein complex, with a 1:1 protein/DNA stoichiometry, was separated from unreacted enzyme and oligonucleotide by SDS–polyacrylamide gel electrophoresis and subjected to in-gel digestion with trypsin. Three cross-linked tryptic peptides (nucleopeptides) of hRPA70×dT₃₀ (T₄₃, T₂₈/2₉, and a truncated T₂₄/2₅) were isolated. Combined mass spectrometric and C-terminal proteolysis experiments showed that at least one amino acid in the segment 235-ATAFNE-240 (located in T₂₄/2₅), at least one out of the two residues sequence 269-FT-270 (located in T₂₈/2₉), and at least one from the sequence 383-VSDF-386 (located in T₄₃) were involved in cross-linking. These peptides contained aromatic residues (F₂₃₈, F₂₆₉, and F₃₈₆ respectively) that can form base-stacking interactions with the DNA and were, therefore, most likely to be involved in cross-linking. The results obtained in this study demonstrate that a combination of exhaustive proteolysis and MALDI TOF MS can localize the sites of DNA binding to very short sequences of amino acids. Data so acquired can confirm or amend information obtained from site-directed mutagenesis and X-ray crystallography.

Protein–nucleic acid interactions are involved in many cellular processes including transcription, translation, replication, recombination, and genomic damage repair. Mass spectrometry in combination with photochemical/chemical cross-linking can complement X-ray diffraction and NMR spectroscopy by providing direct information about the structural relationship between proteins and nucleic acids.¹ A strategy for combining low-intensity UV cross-linking and mass spectrometry (MS) was initially demonstrated in 1994 by this laboratory in collaboration with Bennett et al.² Subsequently, laser-induced cross-linking³⁴ and chemical cross-linking⁵ have been used in combination with MS to investigate protein–DNA interactions. Recently the coupling of photo-cross-linking to MS for protein–DNA analysis has seen more frequent usage.⁶⁻¹⁴

When a nucleic acid binding protein is in contact with its substrate, ultraviolet photons can induce certain amino acids to form “zero-length” covalent bonds with nucleic acid bases that are in close contact.¹⁵⁻¹⁷ The fundamental assumption behind this technique is that only amino acids located in the protein’s binding site are capable of forming these bonds. To identify these amino acids, the UV-irradiated nucleoprotein complex is enzymatically digested with trypsin. Three cross-linked tryptic peptides (nucleopeptides) of hRPA70×dT₃₀ (T₄₃, T₂₈/2₉, and a truncated T₂₄/2₅) were isolated. Combined mass spectrometric and C-terminal proteolysis experiments showed that at least one amino acid in the segment 235-ATAFNE-240 (located in T₂₄/2₅), at least one out of the two residues sequence 269-FT-270 (located in T₂₈/2₉), and at least one from the sequence 383-VSDF-386 (located in T₄₃) were involved in cross-linking. These peptides contained aromatic residues (F₂₃₈, F₂₆₉, and F₃₈₆ respectively) that can form base-stacking interactions with the DNA and were, therefore, most likely to be involved in cross-linking. The results obtained in this study demonstrate that a combination of exhaustive proteolysis and MALDI TOF MS can localize the sites of DNA binding to very short sequences of amino acids. Data so acquired can confirm or amend information obtained from site-directed mutagenesis and X-ray crystallography.

digested to produce free peptides and peptides cross-linked to the nucleic acid substrates (nucleopeptides). After separation from the non-cross-linked peptides, the nucleopeptides are analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS to determine their molecular weights. By comparing the mass of a nucleopeptide minus the mass of the attached oligonucleotide to a peptide map of the native protein, one can identify regions comprising peptides that are involved in cross-linking. This straightforward strategy presumes that the chemistry of the photochemical reaction is known. Finally, the nucleopeptides are sequenced by tandem mass spectrometry (MS/MS) in order to identify the particular amino acids attached to nucleic acid bases. In a few instances, a combination of exhaustive enzymatic digestion and MALDI TOF MS can provide enough information to locate the cross-linked amino acids within a nucleopeptide.

The human replication protein A (hRPA) is a heterotrimeric single-stranded DNA-binding protein that is required for numerous processes in DNA metabolism, including DNA replication, repair, and recombination. Originally, hRPA was purified from human cell extracts and identified as a protein required for simian virus 40 (SV40) DNA replication. Various analyses of hRPA indicated that it is a stable complex of three subunits with molecular masses of 70, 32, and 14 kDa (hRPA70, hRPA32, and hRPA14, respectively). Single-stranded DNA (ssDNA) is bound by hRPA with specific polarity and high affinity. It forms a stable complex with DNA that has an occluded binding site size of 30 nucleotides, 24,25 although two other binding modes involving 8–10 nucleotides and 13–14 nucleotides have been reported. Deletion and mutational analyses have identified four domains in hRPA70: an N-terminal domain (residues 1–168), that participates in hRPA–protein interactions, a central DNA binding domain (residues 169–441), a putative zinc-finger motif (residues 481–503), and a C-terminal domain (residues 503–616). Recent data indicate that the zinc ribbon motif and the C-terminal domain of hRPA70 also compose a DNA-binding domain (domain C). The structure of the central DNA-binding domain has been previously investigated by X-ray diffraction, site-directed mutagenesis, heteronuclear NMR spectroscopy, and fluorescence spectroscopy. The crystal structure revealed that the central DNA-binding domain comprises two three-dimensionally identical copies of a ssDNA-binding subdomain. These subdomains (identified as A and B) resemble an OB-fold motif that has been previously found in various oligonucleotide/oligosaccharide-binding proteins. Subdomains A (residues 181–290) and B (residues 300–426) are oriented in tandem, and a DNA-binding channel extends from one subdomain to the other. Each subdomain is in direct contact with three nucleotides while two nucleotides fill the space between the subdomains. Two primary types of interaction were observed to occur between the protein and DNA: (1) stacking interactions between several aromatic amino acids (F238, F269, W361, F386) and individual nucleic acid bases and (2) hydrogen bonding between amino acid side chains and either the phosphate backbone or individual nucleic acid bases. The X-ray data do not make clear what the individual contributions of these interactions are to the overall DNA-binding affinity of hRPA. Furthermore, experiments with site-directed mutants of hRPA70 call into question the importance of the aromatic amino acids in the protein’s interaction with ssDNA; specifically, when F238 and W361 were individually changed to alanine or when F269 and F386 were simultaneously mutated to alanine, only slight changes in the protein’s binding constant were observed. A double mutant, in which F238 and W361 were changed to alanine, resulted in a decrease of the hRPA binding constant by 3 orders of magnitude; however, proteolytic sensitivity experiments showed that the structure of the doubly mutated protein was disrupted. Structural perturbation makes it difficult to draw conclusions about the double mutant’s phenotype, but the results obtained with the single aromatic residue mutants suggest that no single aromatic residue is critical for the formation of the hRPA–DNA complex. Recent studies suggest a dynamic mechanism for hRPA binding to ssDNA, involving all three domains from hRPA70 (A–C) and a fourth domain from hRPA32 (residues 43–171). This mechanism accounts for the different binding modes observed for hRPA.

In this study, photochemical cross-linking and MS were used to investigate the DNA-binding domain of hRPA. The UV-cross-linking/MS protocol was extended by using proteolysis with carboxypeptidase Y (CPY) in conjunction with MALDI TOF MS. Specifically, this technique was used to locate very short segments of primary sequence in the ssDNA-binding domain of hRPA that contain amino acid residues known to be critical for the formation of a stable protein–DNA complex.

**EXPERIMENTAL SECTION**

**Chemicals.** Acetic acid (AA), trifluoroacetic acid (TFA), ampicillin, bromphenol blue, calcium chloride, copper(II) chloride, fluorinated solvents, and solvents for purification of oligonucleotides.

**EXPERIMENTAL DETAILS**

**Chemical Synthesis.** Oligonucleotides were obtained from Integrated DNA Technologies, Inc.

**UV-ESI-MS.** UV spectra were recorded with a Beckman DU 210 spectrophotometer.

**MALDI-TOF MS.** MALDI-TOF mass spectra were recorded using a Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA).

**RPA Assay.** RPA was assayed as described previously. 

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ride, chloramphenicol, ethylenediaminetetraacetic acid (EDTA), ferric chloride hexahydrate, formaldehyde, formic acid, iodacetamide, inositol, leupeptin, HEPEs, Nonidet P-40, phenylmethylsulfonyl fluoride (PM SF), potassium ferricyanide, sodium chloride, sodium thiocyanate, Trizma (Tris-base), and 2,4,6-tri-hydroxyacetophenone (THAP) were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide (>99% pure), ammonium persulfate, Coomassie Brilliant Blue G-250, 2-mercaptoethanol, bis(N′-methyl-bis-acrylamide, and N,N,N′,N′-tetramethyl-ethylenediamine (TEMED) were obtained from Bio-Rad (Hercules, CA). Ammonium sulfate, dithiothreitol (DTT), glycine, isopropyl diamin (TEMED) were obtained from Bio-Rad (Hercules, CA). Glyceraldehyde and potassium phosphate (dibasic and monobasic salts) were purchased from Fisher Scientific (Pittsburgh, PA). Glycerol and potassium phosphate (dibasic and monobasic salts) were from Becton Dickinson (Franklin Lakes, NJ). Water was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). Dickinson (Franklin Lakes, NJ). Agar, tryptone, and yeast extract were supplied by Becton Dickinson (Franklin Lakes, NJ). Water was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). Glycerol and potassium phosphate (dibasic and monobasic salts) were purchased from Fisher Scientific (Pittsburgh, PA). Glycerol and potassium phosphate (dibasic and monobasic salts) were from Becton Dickinson (Franklin Lakes, NJ). Water was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). Oligodeoxythymidine dT (Grand Island, NY). High-performance liquid chromatography (HPLC) grade acetonitrile, ammonium acetate, ammonium bicarbonate, ammonium hydroxide (50%), glucose, hydrochloric acid, 2-propanol, methanol, potassium chloride, sodium carbonate, sodium hydroxide, sodium nitrate, and sodium thiosulfate were bought from Fisher Scientific (Pittsburgh, PA). Glycerol and potassium phosphate (dibasic and monobasic salts) were purchased from Fisher Scientific (Pittsburgh, PA). Glycerol and potassium phosphate (dibasic and monobasic salts) were from Becton Dickinson (Franklin Lakes, NJ). Water was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). Oligodeoxythymidine dT30, was synthesized by the Biopolymer Core Facility, at the University of Maryland at Baltimore and further purified by native polyacrylamide (15%) gel electrophoresis.

**Protein Expression and Purification.** Recombinant hRPA protein was expressed in Escherichia coli cells by using the T7 expression system.42 E. coli strain BL21(DE3)/pRIL was transformed by electroporation with plasmid p11d-hRPA, obtained from Dr. Mark Wold (Iowa State University).43 Recombinants were selected on LB plates containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). One single recombinant colony was selected to inoculate 1 L of TB medium (1.25 tryptone, 2.4% yeast extract, 0.4% (w/v) glycerol, 0.017 M KH2PO4, and 0.07 M K2HPO4) containing 1% glucose, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol. The culture was incubated overnight at 30 °C without shaking and then placed on an orbital shaker and agitated at 37 °C. When the absorbance at 600 nm was between 0.6 and 0.8, the culture was induced by adding IPTG to 0.3 mM. After 2 h of incubation with shaking, the cells were harvested by centrifugation (4000g) and stored at −80 °C.

All purification steps were carried out at 4 °C. The cell pellet was resuspended in 50 mL of HI buffer (30 mM HEPEs (pH 7.8), 1 mM DTT, 0.25 mM EDTA, 0.25% (w/v) inositol, and 0.01% (w/v) Nonidet P-40) that contained 50 mM KCl, 1 mM PM SF, and 0.1 μg/mL leupeptin. The cells were disrupted by sonification, and the resulting lysate was cleared by centrifugation (30000g for 30 min). The supernatant was applied to a 20-mL Affi-Gel Blue (BioRad) column (1.77 cm2 × 12 cm) equilibrated in HI buffer containing 50 mM KCl. The column was washed sequentially with three column volumes each of HI buffer containing respectively 0.05 M KCl, 0.8 M KCl, 0.5 M NaSCN, 1.0 M NaSCN, and 1.5 M NaSCN. The peak of elution (represented by those fractions that were enriched for the 70-, 32-, and 14-kDa subunits of hRPA) was determined by SDS–polyacrylamide (8%) gel electrophoresis, and the protein bands were visualized with Coomassie stain. Fractions containing the elution peak (present in the column washings with HI buffer containing 1.5 M NaSCN) were pooled and dialyzed (12 000–14 000 MWC SpectraPor dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA)) against HI buffer containing 500 mM NaCl. It is important to note that dialysis into HI buffer containing 250 mM NaCl resulted in extensive protein precipitation. After 6 h, the dialysis buffer was exchanged and dialysis was continued for an additional 14 h. The dialyzed sample was loaded onto a ssDNA agarose (Bennett and Mosbaugh, 1992) column (4.91 cm2 × 4.5 cm) equilibrated in HI buffer containing 500 mM NaCl. The column was washed with three column volumes of HI equilibration buffer and then eluted with a 100-mL linear gradient from 500 mM to 3 M NaCl in equilibration buffer. The hRPA eluted between 0.7 and 1.7 M NaCl, as determined by SDS–polyacrylamide gel electrophoresis. Fractions containing heterotrimteric hRPA were pooled and concentrated by using a Centriprep-30 cartridge (Millipore), and buffer exchanged into HI buffer containing 200 mM KCl. Typically, ~1.0 mg of purified hRPA was obtained per liter of cultured cells; the purity was determined by densitometry of the Coomassie-stained SDS–polyacrylamide gel to be greater than 90% (data not shown). An electrophoretic mobility shift analysis of the purified protein, using [32P]dT30 as the DNA substrate, showed that 99.5% of the purified protein was capable of binding this substrate (data not shown).

**Photochemical Cross-Linking Reaction.** A 1:1 mixture of purified hRPA (800 pmol) and dT30 (800 pmol) in HI buffer containing 200 mM KCl was placed in a 4-mL, capped quartz cuvette (NSG Precision Cells, Farmingdale, NY) and equilibrated at room temperature for 30 min. The cuvette was placed lengthwise on a bed of ice and irradiated with UV light (λmax = 254 nm at a distance of 12 cm) for 1 min in a Stratalinker 1800 cross-linker (Stratagene, La Jolla, CA).

**Isolation of Tryptic Peptides Cross-Linked to dT30.** Ten aliquots of the irradiated cross-linking mixture were loaded onto a SDS–polyacrylamide (8%) gel and subjected to electrophoresis. After removing the stacking gel, the lower 3-cm portion of the resolving gel corresponding to the region of free dT30 was removed, and the remaining portion of the gel was silver stained.44 Bands containing the putative hRPA70×dT30 (XL-1 in Figure 1) were cut from the gel with a clean, sharp razor blade and placed into 1.5-mL Eppendorf tubes. The nucleoproteins were digested in their respective gel slices with trypsin (36 ng/μL) following the procedure of Shevchenko et al.44 After incubation for 2 h at 37 °C, 400 μL of digestion buffer was added to the gel slices, and digestion was continued overnight. Upon completion of digestion, the aqueous solution was removed and saved. An organic extraction mixture of FAPH (50% formic acid, 25% acetonitrile, 15% isopropanol alcohol, and 10% water) was added to each tube until the gel pieces were covered; the tubes were then vortexed at room temperature for 4 h. The extraction mixture was removed from each tube and saved. Gel slices were then crushed and dehydrated with 200 μL of acetonitrile for 10 min. The acetonitrile was removed, care being taken not to remove any gel pieces, and added to the saved FAPH extraction solutions. The

(42) Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. Methods Enzymol. 1990, 185, 60–89.
combined organic solutions were concentrated to near dryness by vacuum centrifugation. The aqueous digest saved earlier was adjusted aqueous digestion solution and the resuspended organic solutions were concentrated to near dryness by vacuum centrifugation. The aqueous digest saved earlier was adjusted aqueous digestion solution and the resuspended organic solutions were concentrated to near dryness by vacuum centrifugation.

**Nuclease P1 Digestion.** Nuclease P1 (ICN Pharmaceuticals, Costa Mesa, CA) was diluted with 50 mM ammonium acetate (pH 6.5) to 0.04 activity unit/μL. After mixing 1 μL of nuclease P1 solution with 1 μL of nucleopeptide sample, digestion was allowed to proceed for 4 h at 37 °C.

**Carboxypeptidase Y Digestion.** Carboxypeptidase Y (Sigma Chemical Co., St. Louis, MO) was diluted with 25 mM ammonium acetate (pH 6.5) to 0.5 activity unit/μL. Extensive digestion was conducted for 48 h at 37 °C after mixing 1 μL of CPY solution with 1 μL of nucleopeptide sample.

**ZipTip Protocol.** The nucleopeptides were purified and concentrated on C18 ZipTips (Millipore) following the manufacturer’s directions for peptides. Metal chelate ZipTips (Millipore) were used to purify and concentrate the CPY-digested nucleopeptide sample before recording its MALDI spectrum. Briefly, 4 μL of 0.1% AA was added to 1 μL of nucleopeptide sample. The ZipTip was preconditioned twice with 10 μL of 0.1% TFA in 50% acetonitrile and then rinsed 10 times with 10 μL of 200 mM ferric chloride hexahydrate in 10 mM HCl, 10 μL of water (Millipore), and 10 μL of 1% AA in 10% acetonitrile. The sample was loaded on the tip by pipetting the solution 20 times in and out of the tip. The tip was also rinsed 5 times with 10 μL of 0.1% AA in 50% acetonitrile and 10 μL of water. Nucleopeptides were eluted with 1 μL of 2% ammonium hydroxide.

**NanoLC Column Preparation.** For on-line nano liquid chromatography (nanoLC) electrospray ionization (ESI) MS, a fused-silica column with an integral frit, PicoFrit (360 μm o.d. × 75 μm i.d. × 40 cm, 15 μm tip) obtained from New Objective (Cambridge, MA) was packed using the pressurized bomb method described by Kennedy and Jorgenson. Briefly, a 40-cm-long PicoFrit column was packed with 5 μm, 300-Å pore, Luna C18 silica gel particles (Phenomenex, Torrance, CA). The packing material suspended in 2-propanol (25 mg/mL) was forced into the capillary by pressurizing the bomb to 1500 psi (~6 h is required to pack a 40-cm-long column). After the gas pressure was slowly released from the bomb, the inlet of the column was connected to an HPLC pump and flushed with acetonitrile for 4 h and Milli-Q water for another 4 h.

**NanoLC Solvent Delivery System.** The nanoLC system employed uses an exponential dilution method to produce gradient separations. A detailed description of the system’s operating principle and setup is provided elsewhere. Briefly, elution gradients are produced by exponential dilution in two mixing chambers. Two conventional HPLC pumps (Kratos Analytical Spectroflow 400, Ramsey, NJ) were used, one to deliver solvent A (0.1% AA plus 0.01% TFA) and the other to deliver solvent B (0.1% AA plus 0.01% TFA in 50% acetonitrile). From the pump, solvent is passed through an in-line filter (Kratos Analytical) into a flow-splitter. The filter helps avoid clogging of the system downstream. The flow-splitter comprises a PEEK Y-piece (Valco, Houston, TX), a 30 μm i.d. × 50 cm fused-silica waste line that functions as a flow resistor, and a 60 μm i.d. × 5 cm PEEK connecting line to the first mixing chamber. The split ratio of this arrangement is between 1:50 and 1:100 so the flow through the analytical column is in the range 300–800 nL/min. A ZDV PEEK union (Upchurch Scientific, Murrieta, CA) drilled out to an internal volume of 3 μL was used as the first mixing chamber of the exponential gradient elution system, and the inlet port of the injection valve served as the second mixing chamber. A micro-injection valve with a port volume of ~10 μL (Rheodyne model 8125, Rohnert Park, CA) and a sample injection loop of 5 μL was used. PEEK tubing with 60-μm i.d. was used to connect the pumps to the filter, splitter, mixing chamber, and injector. The nanoLC column was connected directly to the injector, which was placed in a plastic box, and the spraying voltage (2.5 kV) was applied directly to the injector after loading the sample since the fused-silica needle was not electrically conductive.

Samples were loaded from the injection valve onto the nanocolumn by connecting pump A to the system and raising the inlet pressure to 200 bar so the sample was displaced from the injection loop by solvent A onto the column at a flow rate of 0.8
μL/min. After allowing sufficient time to completely displace the sample from the injection loop (~15 min), pump A was stopped and, after waiting for the pressure to drop to 1 bar, disconnected from the system. Pump B was then connected to the system, and the inlet pressure was raised to 120 bar to deliver solvent B at 0.3 μL/min.

Mass Spectrometry. MALDI TOF MS analyses were performed on a custom-built instrument equipped with delayed extraction and an ion mirror. The MALDI probe was spotted with 1 μL of a 1:3 (v/v) mixture of sample and a saturated solution of THAP in 50% acetonitrile. The instrument was operated in the positive ion mode with an accelerating potential of 20 kV and an extraction delay of 500 ns. A spectrum was produced by using Mo verZ software to collect and average data generated from 30 laser pulses (Genomic Solutions Inc., http://www.genomicsolutions.com).

On-line nanoLC ESI MS analyses were performed on an LCQ ion trap (Thermo Finnigan, San Jose, CA) mass spectrometer. The LCQ was operated in the ESI mode with the spraying potential set to 2.5 kV (applied to the injector), the temperature of the heated inlet capillary at 180 °C, the capillary potential at 46 V, the tube lens offset-potential at 30 V, and a maximum injection time of 50 ms. The instrument was set to acquire a full MS scan between 400 and 2000 m/z followed by an MS/MS scan. For operation in the MS/MS mode, the maximum injection time was increased to 500 ms, the isolation width was set to 2 Da, and the relative collision energy was set to 35% with a 30-ms activation time. Using a data-dependent algorithm, the first most intense ion from a list of parent ions was selected for MS/MS providing the ion’s signal was higher than 2 × 10^4 counts.

RESULTS

In previous studies that have employed a combination of photocross-linking and MS to investigate protein–DNA interactions, photoaffinity labeling was used almost exclusively either with low-intensity UV irradiation or with laser-induced cross-linking. In these earlier experiments, photosensitive nucleobase analogues (5-bromouracil or 5-iodouracil in most cases) were used instead of natural nucleobases in order to increase the cross-linking efficiency. Typically, these analogues are irradiated at wavelengths longer than 300 nm, far beyond the absorbance bands of nucleic acids and proteins, to decrease protein and DNA photodegradation. These nonnatural nucleobases can structurally perturb the native nucleoprotein complex and may photochemically bind to amino acids that are not biologically involved in DNA binding. For this reason, photoaffinity-labeled DNA substrates were not used in this work.

The size of the dT30 substrate used in the present experiments was selected to correspond to the reported size of hRPA’s DNA-binding domain, and its poly(deoxythymidine) composition was chosen because the thymine’s photo-cross-linking reactivity is much greater than that of any other nucleic acid base. Several small-scale electrophoretic mobility shift assay experiments were conducted in order to optimize the ratio of dT30 to hRPA needed to form a stable protein–DNA complex (data not shown). Small-scale UV-cross-linking experiments were also performed to optimize the irradiation time. These cross-linking experiments (data not shown) clearly indicated hRPA70 as the major subunit involved in cross-linking to dT30, a finding in agreement with results obtained by others.43

Preparative-scale cross-linking reactions were performed to obtain sufficient quantities of cross-linked material for characterization of the oligonucleotide-binding domain of hRPA70. Aliquots were removed from the cross-linking mixture both before and after UV irradiation and analyzed by SDS–PAGE (Figure 1). The three bands present in the nonirradiated sample (lane 1) correspond from bottom to top, respectively, to hRPA32, a proteolytic fragment of hRPA70, and hRPA70. No nucleoproteins were present in lane 1 because the noncovalent complexes present in the reaction mixture were disrupted by the denaturing conditions of SDS–PAGE. These three bands were also present after UV irradiation (lane 2) along with two new bands labeled XL-1 and XL-2. Given the appearance of these additional bands after UV irradiation and the fact that their position in the gel was retarded relative to hRPA70, these bands were thought to contain nucleoprotein complexes formed by covalent cross-linking of the hRPA70 subunit to dT30. The XL-1 band that appears at ~80 kDa on the relative molecular weight scale was thought to contain a hRPA70×dT30 nucleoprotein complex while the XL-2 band that appears at ~90 kDa was presumed to correspond to a hRPA × (dT30)2 nucleoprotein. In-gel tryptic digestion followed by MALDI mass mapping of the peptides confirmed the presence of hRPA70 in the XL-1 and XL-2 bands (data not shown). Based on the intensities of the gel bands shown in Figure 1, the cross-linking yield was estimated to be ~50% with respect to hRPA. This study focused only on the analysis of the XL-1 band, leaving the more difficult dinucleotide complex for future study.

The band corresponding to XL-1 from each of the 10 aliquots of the preparative-scale reaction mixture was cut out and individually subjected to in-gel digestion with trypsin; the resulting 10 digests were then pooled. Cross-linked tryptic peptides (nucleopeptides) were separated from non-cross-linked peptides using a procedure based on anion exchange chromatography developed in the authors’ laboratory (see Experimental Section). Identification of the nucleopeptides following in-gel digestion by the procedure described in the Experimental Section was essential in order to observe them by MALDI mass spectrometry. MALDI mass spectra of samples prepared in this manner consistently showed seven prominent ion signals (Figure 2). The three ion signals at m/z 737.4, 1119.6, and 1626.8 corresponded to protonated, non-carboxylic acid, and XL-2 bands (data not shown). Based on the intensities of the gel bands shown in Figure 1, the cross-linking yield was estimated to be ~50% with respect to hRPA. This study focused only on the analysis of the XL-1 band, leaving the more difficult dinucleotide complex for future study.

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Previous mass spectrometric cross-linking studies performed in the authors’ laboratory using poly(dT) substrates established that the masses of nucleopeptides produced by UV-cross-linking are equal within a mass unit to the sum of the neutral oligonucleotide’s mass plus the neutral peptide’s mass. This experimental finding is consistent with the prediction that “zero-length” covalent bonds form between DNA and proteins after low-intensity UV irradiation. Subtracting the average molecular weight of dT30 (9064 Da) from the experimental m/z ratios

three nucleopeptides (nuclease digestion products by MALDI MS (Figure 3) revealed the molecules’ oligonucleotide component. Characterization of the further digested with nuclease P1 in order to reduce the size of whose signals are observed in the same spectrum (Figure 2).

The signals at m/z 1363.8, 1746.0, and 2253.2 were not detected when the same isolation procedure was performed on non-cross-linked hRPA bands. Subtracting the average molecular weight of dT2 (626.4 Da) from the signals observed at m/z 1363.8, 1746.0, and 2253.2 gave the numbers m/z 737.4, 1119.6, and 1626.8, which corresponded respectively to the m/z ratios of the same three protonated, non-cross-linked peptides deduced from the MALDI mass spectrum of the nucleopeptides recorded prior to nuclease digestion (Figure 2).

A portion of nucleopeptide sample digested with trypsin and nuclease P1 was analyzed on a nanoLC ESI mass spectrometer system that was specially constructed to preserve the chromatographic resolution provided by the nanoLC column and to avoid the additional dead volumes that usually accompany the coupling between a nanoLC column and an ESI spray tip. The reconstructed ion chromatogram (Figure 4) indicated the presence of three abundant peptides in the nuclease P1 digest. The ESI MS/MS spectra of these three peptides (Figure 5) indicated that the peptides correspond to the three free peptides whose signals appear in the MALDI mass spectrum of peptides cross-linked to dT30 (Figure 2). Only one of the nucleopeptides (T28/29) at m/z 1746.0) observed in the MALDI spectrum of the nuclease P1 digest (Figure 3) could be detected by ESI MS. This complex eluted between T43 at ~22.1 min and T28/29 at ~22.6 min (Figure 4). It was surprising that interpretable ESI MS/MS data could be obtained for this nucleopeptide because it produced the weakest of the three nucleopeptide signals observed in the MALDI spectrum of the tryptic digest (Figure 3). The nanoLC ESI MS/MS spectrum of doubly protonated T28/29×dT2 (Figure 6) showed that the peptide backbone had fragmented mainly into b ions and that two of these ions (*b9 and *b8) had a dinucleotide tag (626.4 Da) attached to them. The hRPA nucleopeptide sample was extensively digested with CPY for 48 h, cleaned up by immobilized metal affinity chromatography (IMAC), and then analyzed by MALDI MS (Figure 7). The two strongest signals in the range m/z 9000–12 000 of this spectrum corresponded to free dT30 (m/z 9065) and T43×dT30 (m/z 9802). Five other weaker signals in this envelope of ions presumably correspond to nucleopeptides whose C-termini were truncated by the CPY. Prior to CPY digestion (Figure 2), several signals
were observed in the low-mass region ($m/z$ 500–2000), but no signals were observed in this region after CPY digestion. It is conjectured that the free $^{*}T_{24/25}$, $T_{28/29}$, and $T_{43}$ peptides were completely digested by the CPY.

Figure 5. MS/MS spectra of the hRPA70 tryptic peptides separated by nanoLC (Figure 4). Tandem mass spectra were obtained for (A) a singly protonated ion at $m/z$ 737.4, (B) a doubly protonated ion at $m/z$ 560.4, and (C) a doubly protonated ion at $m/z$ 813.9. The sequence information provided by these mass spectra revealed these peptides, respectively, to be $T_{43}$, $T_{28/29}$, and $^{*}T_{24/25}$ minus its five C-terminal residues ($^{*}T_{24/25}$).
The information contained in the MALDI mass spectra (Figures 2 and 3) was sufficient to tentatively identify two of the three putatively cross-linked peptides of hRPA70. The ion signal at \( m/z 737.4 \) was consistent with the T43 tryptic peptide (383-VSDFGGR-389), and the ion signal at \( m/z 1119.6 \) corresponded to the tryptic fragment T28/29 (264-IANKQFTAVK-273), which contains an intact cleavage site. None of the hRPA tryptic peptides had a mass that would account for the \( m/z \) value 1626.8 that is so prominent in the MALDI mass spectra.

Even though nucleopeptides formed by irradiating a mixture of proteins and nucleic acids with UV light appear to be stable to sample preparation and analysis by MALDI and ESI mass spectrometry, the data from the present study indicate that the bond formed between the peptide and nucleotide under UV irradiation can decompose. This is apparently the first reported incidence of such a finding. The appearance of signals corresponding to non-cross-linked peptides in the MALDI spectra (Figures 2 and 3) suggested that nucleopeptide decomposition may have taken place prior to or during ionization. This possibility is further indicated by the appearance of a weak signal corresponding to free dT30 in the MALDI spectrum of hRPA70 peptides cross-linked to dT30 (Figure 2). The appearance of ion signals corresponding to free peptides in the MALDI mass spectra of the nucleopeptide sample (Figure 2), in the MALDI spectrum of the nuclease P1 digest (Figure 3), and in the nanoLC ESI mass spectra of the nuclease P1 digest (Figure 4) also implies an inherent instability in the complexes formed by UV-cross-linking. Whatever the cause, this instability reduced the nucleopeptide concentration, making it more difficult, on one hand, to perform MS/MS on the nucleopeptides themselves but simpler, on the other hand, to identify the peptide components of the nucleopeptides. Specifically, sequence data obtained from the MS/MS spectra (Figure 5A and B) of the peptides that appear at \( m/z \) 9532, 9717, 9886, 9950, and 10334 were assigned to several C-terminal truncated nucleopeptides (see Table 1 for identification).

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the data in the ESI MS/MS spectrum of the doubly protonated form of the unknown peptide (Figure 5C) conformed to the sequence 235-ATAFNEQVDKFFPL-248, which is that of the tryptic peptide T24/25 minus its last five C-terminal amino acids (*T 24/25).

MS/MS data could not be obtained for the T 43 and the *T24/25 dT2 conjugates, but interpretable MS/MS data were produced from T28/29 dT2. The appearance of b4 and b5 fragments in the latter’s MS/MS spectrum (Figure 6) indicated that no dT 2 is attached to the N-terminal segment 264-IANKQ-268 whereas the appearance of *b8 and *b9 fragments implies that a dT 2 is attached to a residue in the segment 264-IANKQFTA-271. Thus, at least one of the residues in the sequence 269-FTA-271 must be a site of cross-linking. The intense signals produced by the singly protonated (m/z 1119.6) and doubly protonated (m/z 560.4) forms of the free T 28/29 (Figure 6) suggested that the dinucleotide tag was easily lost from this nucleopeptide. This lability of the dinucleotide tag under MS/MS conditions would account for the appearance of only two *b fragment ions in the MS/MS spectrum.

The ion signals at m/z 1245.7 and 623.4 correspond respectively to singly and doubly protonated T28/29 (1118.6 Da) cross-linked to thymine (126.1 Da). The fact that these two m/z values correspond to a molecule whose mass is equal to the sum of the two reactants’ masses provides additional evidence that photo-cross-linking reactions between the nucleic acid base thymine and amino acids result in zero-length covalent bonds.

In instances where chromatography and MS/MS fail to provide desired structural data, a combination of exhaustive proteolytic digestion and MALDI TOF MS can provide information about the sites of cross-linked amino acids in nucleopeptides.7 A protease can be used to remove non-cross-linked residues from the N-terminal (aminopeptidase M) or from the C-terminal (CPY) of a nucleopeptide. Since the proteolysis stops when the enzyme encounters a cross-linked amino acid, the result is a truncated nucleopeptide whose molecular weight can be determined by MALDI TOF. This approach was attempted with CPY in this study because carboxypeptidases have been successfully used in combination with MALDI TOF for C-terminal sequencing,48-50 and CPY, in particular, cleaves nonspecifically all C-terminal residues, including proline. In using this method, one must be aware that the kinetics of enzymatic release for different amino acids varies considerably48 and, furthermore, that it is quite possible for steric interference from the oligonucleotide to limit the extent of the CPY digestion.

It was necessary to use IMAC to purify and concentrate the CPY-digested nucleopeptide sample in order to achieve a mass accuracy from the MALDI spectrum (Figure 7) of ~0.05% in the mass range of 9000–12 000 Da. The separation principle of IMAC is based on electronic interactions between phosphate groups and metal ions (Fe3+ in this case) immobilized (chelated) to a stationary phase. This technique was recently employed for enrichment of phosphorylated peptides from crude peptide mixtures,51 as well as for the isolation of nucleopeptides.9 Instead of using the packed column format, it was found preferable in this study to use metal chelate ZipTips (Millipore) designed for low-volume (1–5 µL) samples.

Table 1 displays all possible average molecular weights of truncated HRPA70 nucleopeptides generated by a C-terminal truncation of the unknown peptide (Figure 5C). The molecular weights in boldface letters correspond to the signals observed in the MALDI mass spectrum of a CPY digested nucleopeptide sample (Figure 7).

| Table 1. Average Molecular Weights of All Possible Protonated HRPA70 Nucleopeptides That Would Be Generated from a Mixture Produced by Systematic Truncation from the C-Terminus a |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| T 24/25 nucleopeptide                          | Average Mass (Da)                               | T 28/29 nucleopeptide                          | Average Mass (Da)                               |
| *T 24/25 x dT30                                | 10691                                           | T 28/29 x dT30                                 | 10184                                           |
| ATAFNEQVDKFFP x dT30                          | 10578                                           | IANKQFTA x dT30                                 | 10056                                           |
| ATAFNEQVDKFF x dT30                           | 10481                                           | IANKQFTA x dT30                                 | 9957                                            |
| ATAFNQVDFK x dT30                             | 10334                                           | IANKQFTA x dT30                                 | 9886                                            |
| ATAFNEQVDK x dT30                             | 10187                                           | IANKQF x dT30                                   | 9785                                            |
| ATAFNQVD x dT30                               | 10059                                           | IANKQ x dT30                                    | 9638                                            |
| ATAFNEQV x dT30                               | 9944                                            | IANKQ x dT30                                    | 9510                                            |
| ATAFNE x dT30                                 | 9717                                            | IANK x dT30                                     | 9382                                            |
| ATAFN x dT30                                  | 9588                                            | IA x dT30                                       | 9268                                            |
| ATAF x dT30                                   | 9474                                            | I x dT30                                        | 9197                                            |
| ATA x dT30                                    | 9327                                            |                                                |                                                 |
| AT x dT30                                     | 9256                                            |                                                |                                                 |
| A x dT30                                      | 9155                                            |                                                |                                                 |

a The molecular weights in boldface letters correspond to the signals observed in the MALDI mass spectrum of a CPY digested nucleopeptide sample (Figure 7).
cleavage. By comparing the masses in Table 1 with the masses determined experimentally from the CPY digest spectrum shown in Figure 7, it was determined that the signal at m/z 9532 matches within experimental error the molecular weight of the protonated T43-derived nucleopeptide VSDF × dT30, the signal at m/z 9886 matches the molecular weight of the protonated T28/29-derived nucleopeptide IANKQFT × dT30, and the signals at m/z 9717 and 10334 conform respectively to the molecular weights of the protonated *T24/25-derived nucleopeptides ATAFNE × dT30 and ATAFNEQVDF × dT30. Although the m/z value is too inaccurate to be conclusive, the signal observed at m/z 9950 could have been produced either by IANKQFTA × dT30 or by ATAFNEQV × dT30.

The experimental evidence obtained from the CPY digestion alone suggests that cross-linking occurred to at least one amino acid from the sequence 383-VSDF-386 located in T43, to at least one residue in the segment 264-IANKQFT-270 in T28/29, and to at least one amino acid in the N-terminus segment 235-ATAFNE − 240 of *T24/25. When combined with the ESI MS/MS data obtained for T28/29 × dT2 (Figure 6), the CPY MALDI signal at m/z 9886 localized cross-linking on one of the two residues in the segment 269-FT-270 out of the peptide T28/29.

Using the data obtained from the CPY digestion in conjunction with the tandem mass spectral data, it is possible to deduce a map of the DNA-binding domain of hRPA70. These data are in good agreement with previous information obtained from X-ray crystallography31,32 and site-directed mutagenesis.30,33 Each of the cross-linked peptides from hRPA70 contains one phenylalanine residue previously identified as being involved in base-stacking interactions: F238 belongs to *T24/25 (235-ATAFNEQVDFKFPPL-248), F269 is located within T28/29 (264-IANKQFTAVK-273), and F386 is found in T43 (383-VSDFGRGGR-389). According to the crystallography data,31 W361 has a weak base-stacking interaction in comparison with the phenylalanine residues (F238, F269, F386); hence, it is not surprising that no mass spectrometric evidence was found for the involvement of W361 in cross-linking.

The three-dimensional structure of an hRPA70 fragment (residues 183–420) can be constructed from crystallography data (Figure 8).31,32 In this picture, helices are represented as spirals and β sheets are represented as ribbons with arrows pointing in the C-terminal direction. All information obtained from hRPA to date by UV-photo-cross-linking and MS is summarized in this figure. Three distinct peptide regions in blue contain at least one cross-linked amino acid. These peptides are located in the DNA-binding subdomain A (269-FT-270 and 235-ATAFNE−240) and in subdomain B (383-VSDF-386). No evidence found in this study indicated that a third hRPA-binding domain (C) participated in binding dT30. The aromatic amino acids contained in the three blue regions are in red. They can form base-stacking interactions with the DNA and, in the authors’ opinion, are the residues most likely to be involved in cross-linking. At this point, one can only speculate that these aromatic amino acids are required for the formation of a stable protein–DNA complex. Additional experiments will be required to confirm this hypothesis and to show whether other amino acids are involved in cross-linking.

CONCLUSIONS
In the study of covalent nucleoprotein complexes produced by photochemical cross-linking, there are numerous instances in which chromatography and MS/MS fail to provide the data needed to identify the amino acids that participate in cross-linking. The results obtained in this study illustrate that a combination of exhaustive proteolysis and MALDI TOF MS can be used to localize the sites of DNA binding to very short sequences of amino acids and that the data can be used to complement information obtained from site-directed mutagenesis and X-ray crystallography.

In the specific case of hRPA, the DNA-binding model used in this study, CPY digestion used in conjunction with MS/MS accurately localized amino acids in hRPA that photochemically bind to single-stranded DNA. This mass spectrometric data for hRPA is consistent with the body of information that already exists for this protein’s DNA-binding domain.

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