Identification of Mammalian Proteins Cross-linked to DNA by Ionizing Radiation

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Ionizing radiation (IR) is an important environmental risk factor for various cancers and also a major therapeutic agent for cancer treatment. Exposure of mammalian cells to IR induces several types of damage to DNA, including double- and single-strand breaks, base and sugar damage, as well as DNA-DNA and DNA-protein cross-links (DPCs). Little is known regarding the biological consequences of DPCs. Identifying the proteins that become cross-linked to DNA by IR would be an important first step in this regard. We have therefore undertaken a proteomics study to isolate and identify proteins involved in IR-induced DPCs. DPCs were induced in AA8 Chinese hamster ovary cells by γ-rays under either aerated or hypoxic conditions. DPCs were isolated using a recently developed method, and proteins were identified by mass spectrometry. We identified 29 proteins as being cross-linked to DNA by IR under aerated and/or hypoxic conditions. The identified proteins include structural proteins, actin-associated proteins, transcription regulators, RNA-splicing components, stress-response proteins, cell cycle regulatory proteins, and GDP/GTP-binding proteins. The involvement of several proteins (actin, histone H2B, and others) in DPCs was confirmed by using Western blot analysis. The dose responsiveness of DPC induction was examined by staining one-dimensional SDS-polyacrylamide gels with SYPRO Tangerine followed by analysis using fluorescence imaging. Quantitation of the fluorescence signal indicated no significant difference in total yields of IR-induced DPCs generated under aerated or hypoxic conditions, although differences were observed for several individual protein bands.

DNA-protein cross-links (DPCs) can be induced by a variety of agents, including ultraviolet light and ionizing radiation (IR), metals and semimetals such as chromium, nickel, and arsenic, various aldehydes, including metabolic by-products, and some important chemotherapeutic drugs such as cisplatin, melphalan, and mitomycin C. Measurements of the amount of DNA damage induced by IR indicate that, in a mammalian cell, a 1-Gy exposure induces 1,000–2,000 damaged bases, 800–1,600 damaged sugars, 500–1,000 single-strand breaks, 200–300 alkali-labile sites, 20–40 double-strand breaks, ~30 DNA-DNA cross-links, and ~150 DPCs (2–4). Studies of DPCs induced by various agents have shown half-lives of hours to days (5, 6). This removal is a reflection of both chemical instability and enzymatic repair processes.

Early studies of the DNA-damaging effects of high/supralethal doses of IR (7–10) demonstrated the induction of DPCs by this agent in aerated mammalian cells. IR has also been shown to induce DPCs in hypoxic mammalian cells and to do so more efficiently (1.5–5.5-fold) than in aerated cells (8, 10–13). In addition, cells that are deficient in some DNA repair factors related to cross-link removal show an increased sensitivity to the cytotoxic effects of IR under hypoxic, but not aerated, conditions (14). An interesting implication of these observations is that abrogating the repair of DPCs might be clinically advantageous in radiation therapy because this could render hypoxic tumor cells more radiosensitive (15, 16). However, there are many questions to answer before such a therapeutic strategy might be realized. For example, how are DPCs repaired? How does the presence of a DPC activate a particular repair pathway? Is there a specific damage-recognition event and, if so, is it protein-specific or is it a general mechanism, such as blockage of the progression of protein complexes that mediate various DNA transactions? What are the biological consequences of un repaired DPCs? Identifying the actual proteins involved in these DPCs may help to answer these questions. As well, high doses (typically >30 Gy) have been used in previous studies to measure DPCs. The effect of oxygen on DPC induction at low, clinically relevant, doses has not yet been examined.

To date, various methods (nitrocellulose filter binding, polycarbonate filter trapping, SDS/K+ precipitation, and others (1)) have been used to quantitate and/or isolate IR-induced DPCs with varying levels of success. Trying to purify DPCs by any method that isolates all cellular proteins is clearly going to be noninformative for protein identification, whereas an approach that isolates DPCs by first isolating the DNA should be useful in combination with protein identification methods such as mass spectrometry (MS). Although MS is a sensitive technique, it is possible that only a small percentage of the ~30,000 proteins in the cell can be significantly cross-linked to DNA, so it is essential to obtain a high yield of these proteins with very little contamination in order to identify them. Accordingly, we developed a novel method for the isolation of proteins covalently cross-linked to DNA that yields a sufficient quantity of protein for MS analysis (17). In this study, we have employed this method to purify IR-induced DPCs from mammalian cells, and we have combined this method with MS identification of the isolated proteins. We have also examined the dose dependence of IR-induced DPCs, and we have confirmed the involvement of some of these proteins in IR-induced DPCs by using immunological techniques.
MATERIALS AND METHODS

Cell Culture—The parental Chinese hamster ovary (CHO) cell line, AA8, was obtained from Dr. Keith Caldecott (University of Sussex, UK) and maintained as a monolayer culture in Dulbecco’s modified Eagle’s α-medium/F-12 medium with 10% fetal bovine serum and 5% penicillin/streptomycin in a humidified 5% CO$_2$ and 95% air atmosphere at 37 °C. The human fibroblast cell line, GM00637, was obtained from American Type Culture Collection (Manassas, VA) and maintained as a monolayer culture in Dulbecco’s modified Eagle’s α-medium/F-12 medium with 10% fetal bovine serum and 5% penicillin/streptomycin as above.

Radiation and Chemical Treatment of Cells—Cells were grown as monolayer cultures to ~85% confluency. Aerated cells were irradiated in 150-mm plastic dishes. To render cells hypoxic (18), the cultures were washed with phosphate-buffered saline and trypsinized. Fresh medium was added to a final volume of 5 ml per 2.4 × 10$^7$ cells, and the cells were transferred to 60-mm glass Petri dishes. The dishes were placed in air-tight aluminum chambers, and the chambers were evacuated using a vacuum manifold. The chambers were then filled with pure nitrogen gas, and the cells were incubated at room temperature in nitrogen for 8 s, with the process being repeated four times. This process was repeated another four times using 8-min incubations, and the cells were then incubated at 37 °C for 20 min to allow them to metabolize residual oxygen (adapted from Ref. 18).

For IR treatments, cells were irradiated in a $^{60}$Co irradiator (Gamma-cell, Atomic Energy of Canada Limited, Ottawa, Ontario, Canada) at a dose rate of 0.1 Gy/s. For formaldehyde treatment, 37% formaldehyde was added to the growth medium to a final concentration of 1%, and the sample was incubated at 37 °C for 1 h.

Quantitation of DNA—The UV absorbance at 260 nm was measured for each sample to determine the DNA concentration after DNA digestion. A value of 32 µg/ml per 1 OD unit was used to calculate the amount of DNA in each sample. The amount of DNA in each sample was determined and used to equalize sample loads within each experiment for SDS-PAGE analysis. The 260/280 nm absorbance ratios were also determined. Ratios of 1.5–1.7 were invariably obtained, indicating that the contribution of protein to the 260 nm reading was insignificant. Also, wavelength scans from 230 to 320 nm were examined to verify the contribution of protein to the 260 nm reading was insignificant. A value of 32 µg/ml for each sample was used to determine the DNA concentration after DNA digestion.

For DNA digestion, digestion buffer (1 ml of 10 mM MgCl$_2$, 10 mM ZnCl$_2$, 0.1 mM sodium acetate, pH 5.0) was added to each sample, and the samples were digested for 1 h at 37 °C with 5 units of DNase I (Sigma) and 5 units of S1 nuclease (Invitrogen). After digestion, the DNA concentration was determined by UV absorbance. The samples were washed with distilled deionized water (3 × 10 ml) and concentrated (to 1 ml) in a 15-ml 5-kDa cut-off Centricon concentrator and reduced to dryness by vacuum centrifugation.

DNAzol-Silica DPC Isolation Method—Again, we followed the method of Barker et al. (17). The method of Elphinstone et al. (20) was used to prepare the silica fines. Silica fines (VWR, Mississauga, Ontario, Canada) were heated to near boiling in 5 mM nitric acid, rinsed three times with distilled water, and resuspended in an equal volume of distilled water. The pH of the solution was adjusted to ~7.0 using 1 M Tris-HCl, pH 8.0, and the silica fines were sedimented, resuspended in an equal volume of deionized water, and autoclaved. After lysing the nuclei with DNAzol, 2 ml of 10 mM Tris-HCl, pH 7.0, at 65 °C was added, and each sample was drawn through a 21-gauge needle three times then through a 25-gauge needle three times to shear the DNA. NaCl was added to a final concentration of 4 M, and the mixture was incubated at 37 °C with shaking for 20 min. Urea was added to a final concentration of 4 M, and the samples were incubated as above. An equal volume of 99% ethanol was added to each sample. The silica slurry was then added (1 ml per 7.2 × 10$^7$ cells), and the samples were gently rocked for 20 min at room temperature to allow for binding. The silica was collected by gentle centrifugation, and the supernatant was discarded. The silica was washed three times in 50% ethanol and collected by gentle centrifugation each time. The DNA was eluted two times using 2 ml of 8 M NaOH at 65 °C for 5 min, and eluates were combined. For DNA digestion, 1 ml of digestion buffer (10 mM MgCl$_2$, 10 mM ZnCl$_2$, 0.1 M sodium acetate, pH 5.0) was added to each sample, and the samples were digested with DNase I and S1 nucleases as above. After digestion, the DNA concentration was determined by UV absorbance. The samples were washed with distilled deionized water (3 × 10 ml) and concentrated (to 1 ml) in a 15-ml 5-kDa cut-off Centricon concentrator and reduced to dryness by vacuum centrifugation.

SDS-PAGE Analysis—Laemmli buffer (Bio-Rad) was added to each sample in amounts determined to equalize the DNA concentration of each sample. Samples were analyzed by one-dimensional SDS-PAGE using 15% separating gels (for MS analysis) or pre-cast 10–20% gradient
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gels (Bio-Rad) in later experiments. In separate experiments, gels were stained using either ammonia-silver nitrate or SYPRO Tangerine. Quantitation of Protein on SDS-Polyacrylamide Gels—SYPRO Tangerine-stained one-dimensional SDS-polyacrylamide gels were scanned using a Typhoon 7400 imager (GE Healthcare). These images were analyzed using the ImageQuant 5.2 software (GE Healthcare) by measuring the fluorescence signal. Known quantities of broad range marker proteins (Bio-Rad) were run on each gel and stained with SYPRO-Tangerine. Representative background regions were subtracted for each band, and marker bands were quantified. Quantitation was based on comparison to the fluorescence signal in the marker protein bands after subtraction of representative background regions. Linear regression analysis was performed using the Prism software and analysis of variance and single factor analyses were performed using Microsoft Excel.

Protein Identification by Mass Spectrometry—Protein bands excised from silver-stained SDS-polyacrylamide gels were reduced with dithiothreitol, carbamidomethylated using iodoacetamide, and digested in-gel with trypsin. The resulting peptides were analyzed on a Bruker REFLEX III time of flight mass spectrometer (serial number FM 2413, Bremen/Leipzig, Germany), and the obtained peptide mass maps were searched against data bases to identify proteins (peptide mass mapping). Furthermore, selected peptides were fragmented on a PE Sciex API-QSTAR Pulsar mass spectrometer (serial number K0940105, MDS-Scix, Toronto, Ontario, Canada) to acquire MS/MS spectra, which contain sequence-specific information, and were then subjected to data base searching (MS/MS ion search) to either confirm the previously acquired results from the peptide mass mapping or to identify proteins if no positive results were obtained by peptide mapping.

The MASCOT search engine (www.matrixscience.com/search_form_select.html) was used for both peptide mass mapping and MS/MS ion searching. Data base search criteria are listed below. Two of the most up-to-date and complete proteome data bases, Swiss-Prot, and NCBInr, were selected in our data base search. Mass tolerance was set according to instrument mass accuracy. For peptide mass mapping, the peptide mass tolerance was set at 0.3 Da. For MS/MS ion searching, the mass tolerance was set at 0.3 Da for both the parent ion and fragment ions. Carbamidomethylation of cysteine was set as a fixed modification, whereas methionine oxidation was set as a variable modification.

Western Blots—Cross-linked proteins isolated by the DNAzol-silica method were resuspended in Laemmli Buffer, equalized for total amounts of DNA, separated on 10–20% gradient gels, and stained with SYPRO Tangerine protein stain. Gels were visualized by scanning with the Typhoon 7400 instrument. Gels were then destained briefly in 10% methanol and transferred to nitrocellulose membranes (Bio-Rad) for 1.5 h at 4℃ and 80 V. Blots were blocked in 5% milk/Tris-buffered saline with 0.5% Tween (TBST) for 1 h at room temperature with gentle agitation. After overnight incubation (4℃) with primary antibody, the blots were washed four times (15 min per wash) in 10 ml of 5% milk/TBST at room temperature. Secondary antibodies were incubated with the blots for 1 h at room temperature with gentle agitation. After the secondary antibody incubation, the blots were washed again as above, with a final wash in phosphate-buffered saline. Blots were developed using chemiluminescent reagents by mixing equal portions of the two reagents and incubating each blot with 1 ml of prepared reagent for 5 min at room temperature. Signals were captured using X-Omat K film (Eastman Kodak Co.). The films were scanned as image files, and the optical densities of the bands in these image files were quantified using the ImageJ software/shareware available from the NIH (www.ncbi.nlm.nih.gov). Alternatively, fluorescent secondary antibodies were used, and the blots were analyzed using a LI-COR Odyssey infra-red imager and the ImageJ software to quantitate the band optical densities. The average relative band intensities were plotted after normalizing the most intense signal of the irradiated samples within each determination as 100%.

Antibodies—Primary polyclonal antibodies to mammalian vimentin, histone H2B, histone H4, actin, coflin, hnRNP, poly(ADP-ribose) polymerase (PARP), and HSP10 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary monoclonal antibody to tubulin was also obtained from Santa Cruz Biotechnology. Primary monoclonal antibody to mammalian (mouse) ninein was generously provided by Dr. Gordon Chan (Department of Oncology, University of Alberta). Polyclonal anti-polynucleotide kinase (PNK) antibody has been described previously (21). Primary antibodies to hnRNP A3 and C1/C2 were generously provided by Dr. Gideon Dreyfuss (University of Pennsylvania School of Medicine). Secondary antibodies, peroxidase-conjugated rabbit anti-goat and goat anti-rabbit, were also obtained from Santa Cruz Biotechnology. Primary antibodies were used at dilutions of 1:1,000 in 5% milk/TBST in a total volume of 3 ml. Fluorescently tagged secondary antibodies, goat anti-rabbit IR800, rabbit anti-goat 700, were obtained from Rockland Immunologicals (Gilbertsville, PA) and used at a dilution of 1:5,000 in 5% milk-TBST in a total volume of 5 ml.

RESULTS

Isolation and PAGE Analysis of IR-induced DPCs in Hamster and Human Cells—CHO AA8 cells were exposed to 0 or 1 Gy of γ-radiation under either aerated or hypoxic conditions, and DPCs were isolated using the DNAzol-Strip method. Sample volumes were normalized for the amount of DNA isolated, and cross-linked proteins were analyzed using SDS-PAGE and silver staining. Fig. 1 shows a representative composite gel from these analyses. In the unirradiated sample (Fig. 1, aerated 0 Gy) very little protein was detected, confirming that this method successfully strips the DNA of associated proteins and that we are in fact isolating predominantly covalently cross-linked proteins following IR exposure. This level of isolated protein can be compared with that isolated from irradiated cells (Fig. 1, aerated 1 Gy) or from formaldehyde-treated cells (Fig. 1, F) where each lane contains a greater number and intensity of bands than the control.

Fig. 1 shows similar data for hypoxic cells. Compared with aerated cells, more protein was isolated from the hypoxic untreated sample (Fig. 1, hypoxic 0 Gy), indicating that removal of oxygen leads to modestly increased background DPC induction, although the level of protein was still quite low. Several protein bands observed in the hypoxic irradiated samples were not detected or were less intense in the aerated-irradiated samples (Fig. 1, hypoxic 1 Gy).

The IR-induced cross-linking of proteins to DNA was also examined in human GM00637 fibroblasts. Cells received 0 or 4 Gy of IR under either aerated or hypoxic conditions, and DPCs were isolated using the DNAzol-Strip method. Again, analysis of isolated proteins by SDS-PAGE and silver staining (a representative composite gel is shown in Fig. 2) revealed that little protein was isolated from the unirradiated control samples and that a greater number and intensity of bands were observed in the irradiated samples.

Identification of Proteins Involved in IR-induced DPCs in Hamster Cells—To identify cross-linked proteins, protein bands were excised from several gels. Some of the sample bands that were excised for MS analysis are indicated in Fig. 1 (A1–3 and H1–11). Although similar patterns of bands were seen in different experiments, band selection for MS analysis was based on the intensity and sharpness of band staining in an individual gel. The more intensely and sharply stained bands were
As described under “Materials and Methods,” the search engine, Mascot, was used for protein identification by searching MS data against primary sequence data bases. Mascot uses a statistical scoring algorithm, mouse score, to calculate the matching scores that represent the identification significance. According to its calculation, significance thresholds vary between peptides in the search. Therefore, in our results both mouse scores and significance thresholds are considered. MS/MS spectra were also manually inspected to ensure that the identifications were reasonable and confident. In some cases, the score of a single peptide may be low (lower than the threshold), but correlation with other identified peptides from the same protein and peptide mass mapping results increased the confidence of the identification. If the Mascot MS/MS ion search did not yield positive results (due to internal or side chain fragmentation that Mascot does not account for), a potential peptide match was submitted to the MS product (prospector.ucsf.edu/) program, and the theoretical fragments of the peptide were compared with those shown in the MS/MS spectrum.

To date, we have identified 29 proteins involved in IR-induced DPCs (TABLE ONE). TABLE ONE shows the protein identification results as follows: identified proteins, sequences of peptides selected for MS/MS experiments, peptide masses, Mascot scores for MS/MS ion searches, and the corresponding significance thresholds. Some of the identified proteins were from bands excised from the gel shown in Fig. 1. Tubulin α-6 chain was identified by peptide mass mapping and confirmed by manually matching the MS/MS spectrum with the theoretical fragments. Ninein was identified by peptide mass mapping only because of the unavailability of MS/MS instrumentation at that time.

The proteins identified include the following: structural nuclear-matrix proteins, such as actin and vimentin; spliceosome components such as heterogeneous nuclear ribonuclear proteins (hnRNPs) and the poly-A pyrimidine tract binding protein-associated splicing factor; stress-response proteins such as heat-shock proteins HSP10 and the 78-kDa glucose-regulated protein (GRP78); and chromatin-regulatory proteins such as histones. Some of the proteins identified may be cross-linked to DNA specifically under hypoxic conditions, for example, hnRNP A2/B1, histones H3 and H4, and GRP78.

Confirmation of Individual Proteins Involved in DPCs by Western Blot Analysis—Confirmation and relative quantitation of selected individual proteins identified by the MS analyses were performed by Western blotting of DPC samples from both hamster AA8 and human GM00637 cells. DPC samples, prepared as above, were transferred to nitrocellulose membranes. Samples from hamster cells were probed with antibodies to histone H2B (Fig. 3A) or vimentin (data not shown), and human samples were probed with antibodies to actin (Fig. 3B), tubulin (Fig. 3C), or ninein (data not shown). The Western blotting results confirmed the involvement of each of these proteins in IR-induced cross-links. Based on the relative quantitation from data pooled from multiple independent experiments, the cross-linking of actin and histone H2B increased following irradiation under hypoxic conditions but was not significantly increased over background levels under aerated conditions. The opposite was true for the cross-linking of tubulin, which showed an increase in cross-linking following irradiation under aerated conditions, but no significant increase over background after irradiation under hypoxic conditions. As well, actin, histone H2B, and tubulin were each shown to
TABLE ONE

| Protein                                      | Peptides subjected to MS/MS | Peptide mass | Mascot score | Significance threshold | Aerated* | Hypoxic* | Fig.1 |
|----------------------------------------------|----------------------------|-------------|--------------|------------------------|----------|----------|-------|
| Architectural/structural and associated proteins |                            |             |              |                        |          |          |       |
| β-Actin†                                      | HAPPERK                    | 923.50      | 35           | 27                     | 5        | 3        | H7, H8 |
|                                              | AGFGADDAPR                 | 976.45      | 25           | 24                     |          |          |       |
|                                              | VAPEEHVULTEAPLNPK          | 1954.07     | 50           | 12                     |          |          |       |
|                                              | GSYTTTAER                  | 1132.53     | 47           | 15                     |          |          |       |
|                                              | QYEDESQPSIVHR              | 1516.71     | 62           | 21                     |          |          |       |
|                                              | DSYYGDEAQSKR               | 1354.63     | 26           | 15                     |          |          |       |
|                                              | SYELPDQVITIGNER            | 1790.89     | 24           | 12                     |          |          |       |
|                                              | JWHTFYNELR                 | 1515.75     | 16           | 10                     |          |          |       |
|                                              | AVFPSIVGR                  | 945.50      | 35           | 27                     |          |          |       |
|                                              | QYEDESQPSIVHR+Pyro-glu (N-term Q) | 1499.68     | 53           | 15                     |          |          |       |
| Cofilin                                      | EDLVFIFWAPESAPLK           | 1861.98     | 28           | 55                     | 1        |          |       |
|                                              | KEDLVFIFWAPESAPLK          | 1990.00     | 20           | 14                     |          |          |       |
| Vimentin‡                                     | VELQELNDR                  | 1115.57     | 17           | 24                     | 2        | 3        | A1, H5 |
|                                              | MALDIEATYR                 | 1295.67     | 39           | 15                     |          |          |       |
|                                              | EEAESTLQSFQ                | 1296.61     | 26           | 19                     |          |          |       |
|                                              | SLYSSSPGGAYVTR             | 1444.71     | 47           | 20                     |          |          |       |
|                                              | LGDLYEEEMR                 | 1254.57     | 12           | 18                     |          |          |       |
|                                              | QYQLSTCEVDALGKTNESLER      | 2377.17     | 23           | 14                     |          |          |       |
|                                              | SLPLPNSLNLIR               | 1570.90     | 42           | 24                     |          |          |       |
| Tropomyosin, α3 chain and α4 chain            | KYEEVAR                    | 894.46      | 37           | 30                     | 2        | 1        | A3, H11|
|                                              | KIQVQ0QADDAEER             | 1770.90     | 50           | 22                     |          |          |       |
|                                              | VTIAGQ6GVLPIQAVLPPK        | 1931.17     | 18           | 14                     | 1        |          |       |
| Histone H2A‡                                  | VTIAQGGVLPNIQAVLPPK        | 1931.17     | 18           | 14                     | 1        |          |       |
| Histone H2B‡                                  | AMGIMNSFVNDIFER            | 1743.82     | 38           | 16                     | 1        | 3        |       |
|                                              | AMGIMNSFVNDIFER 2Oxidation(M) | 1775.81     | 23           | 16                     |          |          |       |
|                                              | STITSREIQTVAYR             | 1461.80     | 34           | 61                     |          |          |       |
| Histone H3‡                                   | KLPFQR                     | 788.48      | 22           | 31                     | 3        |          |       |
|                                              | STELLIR                    | 831.50      | 46           | 31                     |          |          |       |
|                                              | YQKSTELLIR                 | 1250.71     | 43           | 21                     |          |          |       |
|                                              | EIADQFKTLDR                | 1335.69     | 159          | 55                     |          |          |       |
|                                              | YRPGTVALR                  | 1032.60     | 5            | 16                     |          |          |       |
|                                              | KPHYRPGTVALR               | 1550.91     | 188          | 55                     |          |          |       |
| Histone H4‡                                   | VELENVIR                   | 989.58      | 39           | 30                     | 3        |          |       |
|                                              | SGLIYEETR                  | 1180.62     | 33           | 28                     |          |          |       |
|                                              | DNOQGKTPAIR                | 1325.76     | 30           | 22                     |          |          |       |
| CGI-55 protein                               | AKVEFNIR                   | 976.56      | 23           | 28                     | 1        | H5       |       |
|                                              | REEFKPLEEK                 | 1175.64     | 26           | 25                     |          |          |       |
|                                              | RPQDQQLQEGK                | 1255.64     | 40           | 23                     |          |          |       |
| Nuclease-sensitive element-binding protein 1 | RPENPKFQDGK                | 1265.66     | 62           | 29                     | 1        | H6       |       |
|                                              | REPENPKPQDGGK              | 1421.76     | 10           | 13                     |          |          |       |
| PTBP-associated splicing factor              | REPENPKQDGK                | 1421.76     | 10           | 13                     |          |          |       |
|                                              | NEQGESEAPSEQQAQQR          | 1587.70     | 46           | 29                     |          |          |       |
|                                              | FQGGAGPGVGGQGPR            | 1341.67     | 53           | 31                     |          |          |       |
|                                              | FAQHGTFFYESQR              | 1762.78     | 20           | 16                     |          |          |       |
|                                              | GIVEFASKPAAR               | 1245.70     | 18           | 22                     |          |          |       |

*Note: most peptides were detected in multiple experiments, and modified peptides were also detected (oxidized, acetylated, etc.).
cross-link to DNA when cells were treated with formaldehyde, which is a well known DPC inducer.

Proteins identified to be involved in IR-induced cross-links by MS but that were negative on all Western blots attempted (n/H110059) were the hnRNP A/B proteins; this may be due to antibody quality or masked/destroyed epitopes, although most of the proteins examined in our analyses showed no size reductions/proteolysis (i.e. they migrated at the expected sizes).

Identification of Additional Novel Proteins Involved in DPCs by Western Blot Analysis—Our list of proteins involved in IR-induced DPCs is not exhaustive, and no doubt further MS analyses would lead to the identification of additional proteins. Because our MS data indicated the involvement of hnRNP A2/B1 and A3 in IR-induced DPCs, we probed for the related proteins hnRNP C1/C2 (Fig. 4A). Western blotting demonstrated the involvement of these splicing components in IR-induced DPCs as the level of protein cross-linked to DNA increased after irradiation under both aerated and hypoxic conditions.

Given that any proteins that are frequently in contact with the DNA, such as DNA repair proteins, are potential targets for cross-linkage by IR, we investigated the involvement of several additional proteins in IR-induced DPCs by Western blotting. Both PARP (Fig. 4B) and PNK (Fig. 4C) were found to be involved in IR-induced DPCs in human cells. Based on relative quantitation from data from multiple independent analyses, PARP and PNK each demonstrated an increased cross-linking to DNA after irradiation under hypoxic conditions. PARP demonstrated a more marked increase in cross-linking after irradiation under aerated conditions, whereas PNK showed a background level of DPC induction under aerated conditions. The PNK and hnRNP C1/C2 proteins also showed increased cross-linking after treatment of cells with formaldehyde, but PARP was not significantly different from background.

Quantitation of IR-induced DPCs in Mammalian Cells—As shown above, Western blotting allowed for the relative quantitation of cross-linking of individual proteins to DNA. The next step was to examine the dose dependence of total DPC induction by IR. The DNAzol-Strip
method is useful for isolating high yields of cross-linked proteins (which is important for MS), but previous work with topoisomerase poison-induced DPCs (17) indicated that the lengthy processing time and conditions may result in the loss of some DPCs. For quantitation work, we switched to the DNAzol-Silica method as it is more rapid, thus reducing the opportunity for DPC reversal or degradation. We also switched from silver staining (Figs. 1 and 2) to SYPRO Tangerine staining (Figs. 5–8) because the latter method has a lower detection limit, low protein-to-protein staining variability, and is quantitative, whereas silver staining is not. Fig. 5, A and B, shows typical SYPRO Tangerine-stained SDS-PAGE analyses of cross-linked proteins from aerated (Fig. 5A) and hypoxic (Fig. 5B) CHO AA8 cells exposed to increasing doses (0–4 Gy) of γ-ray radiation. Again, the stringency of the removal of non-cross-linked proteins was confirmed by examining the lane for the unirradiated aerated cell sample, which contained little protein. The extent of cross-linking was quantified for the total protein for each sample (Fig. 5C) by using known quantities of the size-marker proteins for calibration and the ImageQuant 5.2 software on the Typhoon 7400 imager and subtracting a representative background region for each sample. This was performed on seven independent experiments. Under aerated conditions, there appeared to be a dose-responsive cross-link induction that plateaued at 2 Gy. This was not the case under hypoxic conditions where the induction of DPCs with increasing doses of radiation

FIGURE 3. Confirmation and quantitation of individual proteins in IR-induced DPCs. Western blot analysis and quantitation of histone H2B (A), actin (B), and tubulin (C) in IR-induced DPCs in hamster (A) and human cells (B and C). Quantitations were performed on pooled data as described under “Materials and Methods.”

FIGURE 4. Identification and quantitation of additional proteins in IR-induced DPCs. Western blot analysis and quantitation of hnRNP C1/C2 (A), PARP (B), and PNK (C) in IR-induced DPCs in hamster (A and B) and human cells (C). Quantitations were performed on pooled data as described under “Materials and Methods.”
approached linearity ($p = 0.077$), but possibly reaching a plateau by the higher doses.

We then analyzed the dose dependence of IR-induced cross-linking in these cells in a lower dose range, 0–1.5 Gy (Fig. 6). Representative gels of isolated DPCs from aerated and hypoxic cells are shown in Fig. 6, A and B, respectively. The statistical analyses were carried out on pooled data ($n = 10$) and demonstrated that there is a linear dose-responsive relationship for DPC induction in aerated and hypoxic cells below 2 Gy ($p = 0.035$ and $0.048$, respectively).

Similar analyses were performed for IR-induced protein cross-linking to DNA in human cells. Fig. 7 shows a typical analysis of cross-linked proteins isolated from normal human fibroblasts exposed to 0–4 Gy of $\gamma$-radiation under aerated (Fig. 7A) or hypoxic (Fig. 7B) conditions. Under aerated conditions, the IR-induced cross-linking of protein to DNA does increase linearly over the 0–4-Gy dose range ($p = 0.042$, $n = 6$), but this linearity was not observed under hypoxic conditions ($p = 0.1$, $n = 6$). Additional analyses were also performed in human cells using a lower dose range (Fig. 8). Representative gels for DPCs isolated from aerated (Fig. 8A) and hypoxic (Fig. 8B) cells exposed to 0–1.5 Gy of $\gamma$-radiation are shown. The results were similar (Fig. 8C) for both aerated and hypoxic conditions, although neither condition revealed a linear dose-dependent induction of DPCs by IR ($p = 0.082$ and $0.089$ ($n = 12$) for aerated and hypoxic cells, respectively).

An advantage of this approach is that SYPRO Tangerine staining and ImageQuant software enable analysis of the cross-linking response of individual protein bands, as shown in Fig. 8D, where we have analyzed the protein content of two sample bands ($H1$ and $H2$ in Fig. 8B). We further compared the quantitation of a specific band with the Western blot for a specific protein constituent of that band (PARP) in an individual experiment (Fig. 9). DPCs isolated from hypoxic CHO AA8 cells exposed to 0–1.5 Gy of $\gamma$-radiation were separated on SDS-PAGE and

![Figure 5](image5.png)

**FIGURE 5. Quantitation of DPCs from hamster cells, 0–4 Gy.** CHO AA8 cells received 0, 1, 2, or 4 Gy of $\gamma$-radiation under either aerated (A) or hypoxic (B) conditions. Lane M represents molecular mass marker proteins with masses shown in kDa. DPCs were isolated using the DNAzol-Silica method and analyzed by SDS-PAGE and SYPRO Tangerine staining. The dose in Gy is indicated above each lane. C, plot of total protein isolated per $\mu$g of DNA for each sample. Means, S.E., and linear regression analysis were performed on data accumulated from seven independent experiments.

![Figure 6](image6.png)

**FIGURE 6. Quantitation of DPCs from hamster cells, 0–1.5 Gy.** CHO AA8 cells received 0, 0.5, 1.0, or 1.5 Gy of $\gamma$-radiation under either aerated (A) or hypoxic (B) conditions. Lane M represents molecular mass marker proteins with masses shown in kDa. DPCs were isolated using the DNAzol-Silica method and analyzed by SDS-PAGE and SYPRO Tangerine staining. The dose in Gy is indicated above each lane. C, plot of total protein isolated per $\mu$g of DNA for each sample. Means, S.E., and linear regression analysis were performed on data accumulated from 10 independent experiments.
stained with SYPRO Tangerine. The total protein in the band migrating with an approximate molecular mass of 116 kDa (Fig. 9A) was quantified (Fig. 9B). After protein transfer to nitrocellulose, the blot was probed with antibodies to PARP, and the signal intensities were determined (Fig. 9, C and D). This result highlights the applicability of this DPC analysis approach to the eventual dissection of the biological relevance of the cross-linking of individual proteins.

DISCUSSION

Several proteins involved in DPCs induced by various agents have been identified previously by using immunological methods (1, 22–29). Early studies of formaldehyde- and IR-induced cross-linking logically focused on histones because of their known close association with DNA, and these proteins were indeed shown to be cross-linkable by both of these agents, although the studies with IR were performed in vitro using cell-free systems. One study (23) did examine the cross-linking of proteins to DNA by IR in vivo and identified three such proteins: actin, lectin, and aminoglycoside nucleotidyltransferase. There is information about a role for actin in the nucleus (30), but the biological relevance of the other cross-linked proteins within the nucleus is unknown.

In this study, we have successfully combined stringent protein removal with SDS-PAGE separation and sensitive MS analysis to identify a set of proteins cross-linked to DNA in mammalian cells following exposure to IR. We have presented here the identification of 29 proteins that appear to be involved in IR-induced DPCs. These proteins fall into several categories according to their nuclear functions. Among these are structural/nuclear matrix proteins such as actin and vimentin, spliceosome components such as hnRNPs and protein-associated splicing factor, stress-response proteins such as HSP10 and GRP78, chromatin regulatory and structural proteins such as histones, as well as proteins involved in other DNA transactions such as the chromatin remodeling protein CGI-55, and proteins whose nuclear functions are not yet fully known. These proteins must be located within several nanometers of the DNA to become cross-linked. This means that such proteins are in direct association with the DNA or in very close proximity at the time of irradiation or shortly thereafter. Some of the proteins identified here have already been shown to be involved in DNA metabolic processes and in association with DNA, for example the histones, whereas others have not. Another factor affecting the likelihood that a protein will be identified as being involved in DPCs is whether it is sufficiently abundant that it can be visualized as a distinct band on SDS-PAGE and in sufficient yield to be identifiable by MS.

The nuclear localization of some of these proteins, for example glyceraldehyde-3-phosphate dehydrogenase and vimentin, was originally discounted as artifactual, and yet further work on such proteins has led to the proposal of new and additional roles in DNA repair and recombination processes (31, 32). Other proteins on our list also have roles in these processes, which may explain their association with DNA (TABLE TWO). A number of mRNA-processing components were identified in this study, and their presence was probably not a result of their cross-linking to ribonucleic acid because DNAzol effectively hydrolyzes RNA. We must therefore consider the possible association of these proteins with DNA directly, and thus what roles they might be performing in this regard. For example, a recent study has shown the involvement of hnRNPA1 in Okazaki fragment maturation during DNA replication as a stimulator of the FEN-1 nuclease (33). A novel role of another ribonucleoprotein, this time in genome maintenance, has also been demonstrated; the TLS/FUS protein has been shown to mediate homologous pairing and to be involved in the DNA damage response (34). The TASR (TLS-associated SR) proteins, identified as being involved in DPCs in our study, may also be involved in these processes because of their association with TLS.
We were not able to find evidence of nuclear localization or nuclear function for every protein that we identified. It may be that the fingerprint peptides used for identification will also be found in novel nuclear proteins. More likely, there may be yet unknown nuclear functions for some of the proteins we have identified. Indeed, new nuclear functions have been found for other proteins. For example, recent studies have shown that the centriole protein, Centrin 2, localizes to the nucleus and binds the nucleotide excision repair protein, XPC, suggesting a link between cell division and DNA repair (35). Similarly, the DNA ligase IV protein has been shown recently to interact with a subunit of the human condensin complex, defining a new link between chromatin structure and DNA repair (36). Identification of proteins as being in close proximity to the DNA may also provide insight into the content and organization of the protein complexes involved in various processes in the nucleus.

Any protein involved in DNA metabolic processes (repair, replication, recombination, etc.) is a potential candidate for involvement in this form of IR-induced lesions. Based on their known association with DNA, we investigated the involvement of two additional DNA-repair proteins, PARP and PNK, in IR-induced DPCs through immunoblotting experiments. The PARP proteins are involved in DNA damage signaling (37, 38), and PNK is involved in strand break repair. Both of these proteins were indeed shown to be cross-linked to DNA in response to IR.

Other important aspects of the measurement of IR-induced cross-linking of proteins to DNA are the background level of cross-links and the dose and oxygen dependence of these events. The SDS-PAGE analysis of DPC isolates demonstrated that the DPC isolation method was sufficiently stringent to strip noncovalent DNA-protein complexes from the DNA based on the low level of protein observed in the unirradiated aerated sample (Fig. 1, 0 Gy sample). As with other forms of DNA damage, DPCs can be induced by endogenous agents (e.g., free radicals generated during normal cellular metabolism). Human cells have been reported to have a base-line level of DPC induction of 0.5–4.5 per 107 bases (39) and a base-line level of thymine-tyrosine DPCs of ~7 molecules/106 bases (40). However, the detection limit of the DPC isolation and measurement methods will have a major impact on this parameter. For example, the alkaline elution/polycarbonate filter method failed to detect DPCs in unirradiated cells, and only detected DPCs in irradiated cells at doses of 50 Gy (41). The nitrocellulose filter binding technique is more sensitive and can detect DPCs in irradiated cells at doses of ~30 Gy (41). The method presented here allowed the detection of DPCs in irradiated cells at biologically relevant doses as low as 0.5 Gy.

Based on previous observations, it has been suggested that molecular oxygen favors DNA fragmentation reactions at the expense of DNA-protein radical reactions following exposure to IR (42). Several previous studies (10, 11, 13, 43) report an increased induction of DPCs under hypoxic conditions; however, these studies typically used much higher doses of radiation and the alkaline elution assay for DPC measurements. By using the alkaline elution assay, the amount of cross-linking is inferred from the extent of DNA retention on filters, which is also influ-
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| Protein | Relevant roles and/or nuclear localization for identified proteins | Roles |
|---------|---------------------------------------------------------------|-------|
| Histone H2A, H4 | DNA organization, modified forms involved in DNA repair (58) |       |
| Histone H2B | DNA organization |       |
| Histone H3 | DNA organization, telomere binding (59) |       |
| CGI-55 protein | Potential chromatin remodeling factor (60) |       |
| Ninein | Microtubule anchoring; localizes to nuclei in interphase (61) |       |
| Tubulin | Component of microtubule organizing center in nucleus (62) |       |
| Vimentin | Chromatin remodeling, recombination (32) |       |
| Actin | Nuclear scaffold, chromatin remodeling (63), regulation of DNA replication and/or transcription (64, 65), senescence marker (66) |       |
| Cofilin | Actin-regulatory protein; nuclear accumulation in senescence (66) |       |
| Elongation factor 1 α1 | Actin organization, transcription; nuclear localization in apoptotic cells (67) |       |
| Tropomyosin | Actin-regulatory protein |       |
| Radixin | Actin-organizing protein, localizes to nucleus (68) |       |
| 78-kDa glucose-regulated protein (GRP78) | Stress response, protein folding; localizes to endoplasmic reticulum (69) |       |
| Calumenin/crocalbin | Ca2⁺-regulatory protein, localizes to the endoplasmic reticulum and Golgi (70) |       |
| α2-Macroglobulin receptor-associated protein | Protein folding, localizes to endoplasmic reticulum (71) |       |
| 10-kDa heat shock protein, mitochondrial (chaperonin10) | Stress response, protein folding, localizes to mitochondria and secretory compartments (72) |       |
| Thioredoxin peroxidase II | Oxidative stress response (73, 74), apoptosis inhibitor (75), maintenance of genome stability (76) |       |
| Serotransferrin precursor | Iron transport, transferrin localizes to endosomes (77) |       |
| TLS-associated SR Protein, TASR-2 | mRNA splicing factor; TLS is nuclear (78, 79) |       |
| Heterogeneous nuclear ribonuclear protein A3 | mRNA splicing factor, nuclear localization (80) |       |
| Heterogeneous nuclear ribonuclear protein A1 | mRNA splicing factor, FEN-1 stimulator (33), telomere formation and/or stabilization (81), control of apoptosis (82) |       |
| Heterogeneous nuclear ribonuclear protein A2/B1 | mRNA splicing factor, telomere formation and/or stabilization (81), control of apoptosis (82) |       |
| 40 S ribosomal protein S24 | Part of hnRNP complex involved in mRNA processing |       |
| Rho GDP-dissociation inhibitor 1 (GDI 1) | Modulator of Rho GTPases; nuclear localization? (83, 84) |       |
| Splicing factor-PTB protein-associated | Homologous pairing promoter (85) |       |
| Nucleosome-sensitive element-binding protein 1 | Recognizes unusual DNA structures (86) |       |
| Glyceraldehyde-3-phosphate dehydrogenase | Repair of thioguanylated DNA (31), transcription (65) |       |

DPCs in hypoxic cells are not detected by this method at doses below 5 Gy (11). By using our new, sensitive method for DPC detection and low doses of radiation (0–4 Gy), we did not observe a dramatic effect of hypoxia on DPC induction. Quantitation of the cross-linking of individual proteins, however, revealed an oxygen dependence of cross-linking for some proteins (e.g. H2B, actin, hnRNPC1/C2, and PNK) but not others (e.g. PARP and tubulin). This suggests that proteins become cross-linked to DNA by different mechanisms. Indeed, it has been shown that there are chemically different forms of cross-links and that the induction of some cross-links is not influenced by the presence of oxygen in vitro (44–47). Proteins can become cross-linked to DNA directly through a process mediated by IR-induced radicals. Because the lifetime of these radicals is very short, only those proteins that are located within several nanometers of the DNA at the time of irradiation can become cross-linked. Alternatively, radiation exposure can potentially produce cross-links on a delayed time scale as a result of the generation of reactive aldehydes or possibly other longer lived species (47, 48).

The dose dependence of DPC induction revealed a linear relationship in hamster cells under aerated and hypoxic conditions in the low dose range (0–1.5 Gy). In human cells, DPC induction under aerated and hypoxic conditions approached linearity in the same low dose range (0–1.5 Gy). At a higher dose range (0–4 Gy), DPC induction in hamster cells appeared to plateau or decline under aerated conditions but approached linearity under hypoxic conditions. The opposite effect was seen in human cells; DPC induction in the 0–4 Gy dose range was linear under aerated conditions but appeared to plateau or decline under hypoxic conditions. A nonlinear dose response for cross-link induction has been reported previously for other genotoxic agents. A decline in damage frequency with dose was reported for the cross-linking of nuclear matrix proteins to DNA by the antitumor agent cisplatin (24). In the cisplatin study, SDS-PAGE indicated that there was little change in the extent of cross-linking of abundant proteins to DNA with cisplatin concentrations between 35 and 1,000 μM. Drug concentration-responsiveness of DPC induction was demonstrated for individual proteins in that study (24) using immunoblot analysis. A similar phenomenon has also been reported for laser-induced DNA-protein cross-linking (49) and for UV-induced recombination (50), although the cause of this effect is not yet known. It is inevitable that cellular DPC induction will be a saturable process given that there are a finite number of proteins per cell in contact with the DNA and available for cross-linking reactions. Also, because of the differing protein abundances, this plateau will occur at different doses and with differing amplitudes for individual proteins. This was demonstrated by the quantitation of individual proteins. It may also be that at higher doses of IR there are other types of damages that are occurring, which may affect either DPC induction or the efficiency of DPC detection.

It should be noted that other studies demonstrating the dose dependence of IR-induced DPCs used higher doses (and therefore longer irra-
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diation times) than the present study. It is known that exposing cells to IR rapidly induces changes in chromatin structure (51). It may also be the case that the stress induced by hypoxia also alters chromatin structure. Also, in the present study we have used unsynchronized cell populations, and there may be variations in the efficiency of chromatin stripping depending on the cell type and growth phase (52, 53). Work from the Oleinick group (54–57) has shown that actively transcribing DNA regions are more susceptible to DPC induction and that cells in metaphase not only display a higher background level of DPCs but also show a more gradual dose response for IR-induced DPCs compared with asynchronous cell populations.

The cross-linking of nuclear proteins to DNA would be expected to have serious consequences for DNA metabolic processes such as the progression of replication, transcription, and repair complexes. Determining which proteins become covalently cross-linked to DNA may help to unravel the consequences of DPCs for these DNA transactions and enable a better understanding of the contribution of different types of DPCs to IR-induced responses such as mutagenicity, transformation, and cytotoxicity. It is not unreasonable to imagine that there may be as yet undiscovered cooperative interactions between the machinery that controls various aspects of the cell cycle and organization of DNA/chromatin and members of the protein complexes that perform replication, recombination, transcription, etc., and/or multiple roles for some of these proteins in different nuclear metabolic processes. The identification of the proteins involved in DCPs should therefore provide useful information toward determining the consequences and repair of these DNA lesions and may also provide some insight into the structural and temporal arrangements of protein complexes in cellular chromatin.

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