Intracellular Levels of Centrin2, XPC, & hHR23B Increase After DNA Damage

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Abstract

Centrin2, Xeroderma Pigmentosum Complementation group C (XPC) & hHR23B are all components of the Global Genomic Nucleotide Excision Repair Machinery (GGNER). These components act to detect defects or damage in the DNA of human cells. This study analyzed the movement of Centrin2, XPC, and hHR23B in two human breast cancer cell lines, MDA-MB 231 and MCF-7, after subjecting them to DNA damage caused by hydroxyurea. Using western blot analysis, it was found that intracellular levels of centrin2, XPC, and hHR23B all increased after DNA damage. It was also found that centrin2 moves out of the cytoplasm and into the nucleus, when DNA damage was induced.

Background

The DNA in a human cell is subjected to thousands of lesions every day (Alberts, et al, 2002). These might include ultra violet radiation damage from the sun, chemical and pH damage from our environment, or even physical damage suffered from trauma. If left untreated, these lesions can lead to single and double strand breaks, improper synthesis of proteins, and even cell death. If the DNA is damaged in specific loci, these lesions can give rise to cancer.

In humans, there are at least seven repair pathways that can correct the damage that has been done to DNA. One of these pathways is called the global genomic nucleotide excision repair pathway, or GGNER. This repair pathway is one of the most versatile, and it can correct bulky DNA adducts and helix distortions such as those resulting from ultra-violet light-induced thymine dimers, or cisplatin-DNA adducts (Dorin, 2005). In GGNER, the XPC protein binds to damaged sections of DNA, followed by Centrin2 and hHR23B binding to XPC. This complex then recruits other proteins which make the necessary repairs and synthesize a new section of DNA in place of the damaged one. If this process does not repair the damaged sections of DNA, a signaling pathway is activated and the cell becomes apoptotic. (Alberts et al , 2002).

The first component of the GGNER is XPC, a 120kD protein that is especially important in the recognition of DNA damage (Sugasawa, 2005). Specifically, XPC recognizes helix distortion lesions and initiates their repair (Nelson, 2005). The importance of XPC in correcting DNA damage is evident in diseases such as Xeroderma Pigmentosum. Individuals with this disease do not express XPC, and as a result, appear to age abnormally fast and have an extreme sensitivity to sunlight. Consequently, people afflicted with Xeroderma Pigmentosum frequently develop skin and organ cancer (Shimizu, 2002).

Another of the components of the GGNER machinery is Centrin2 (Molinier, 2004). This protein is approximately 20kDa and is primarily found in the centrosome and the pericentriolar material. Centrin2 is a calcium binding protein and a member of the calmodulin family. As such, Centrin2 is highly reactive to calcium, an important element many cellular activities. Centrin2 has been found in basal bodies and in the dynien light chain (Young, et al, 2000). In one study (Salisbury, 2002), Centrin2 was also shown to be absolutely necessary for centriole duplication. Using RNA interference, the study demonstrated that the knockdown of Centrin2 resulted in failure of centriole duplication during the cell cycle in HeLa cells. This led to the hypothesis that Centrin2 is a checkpoint protein which controls two intracellular processes following DNA damage. The first process would be centriole duplication, since cells expressing reduced levels of Centrin2 fail cytokinesis and form multi polar spindle bodies. The second is the enhancement of GGNER by stabilizing XPC through the binding of Centrin2 at its C terminal. (Charbonnier, 2007). This study showed that apolar side chains of XPC bind into the calcium terminal of Centrin2 and that when levels of XPC were over expressed in the cell, Centrin2 moved out of the cytoplasm and into the nucleus.

The third DNA damage recognition component in GGNER is the protein hHR23B. This protein is approximately 57kDa and was shown to exist in a 1:1:1 stoichiometric ratio with Centrin2 and XPC (Araki, 2001). hHR23B has a strong affinity for polyubiquinated substrates, and may act to enhance or decrease immune response (Anderson, 2005). In another study (Katyar and Lennarz, 2005), hHR23B was shown to exist in the cytoplasm of a cell and be involved with cell cycling, in the G1-S checkpoint. A third study (Kaur, 2007) showed that hHR23B plays a critical role in the activation and function of p53 after specific genotoxic exposures.

At this time it is known that XPC, Centrin2 and hHR23B are all involved in GGNER and that Centrin2 and hHR23B play a contributing role as cell cycle checkpoint proteins, but how these proteins perform these roles is not completely understood. This paper provides a more clear understanding about how these proteins execute the GGNER process by investigating the amounts and localizations of these proteins both before and after DNA damage has been induced. In order to assess the expression of and localization of XPC, Centrin2, and hHR23B, two cell lines were treated with hydroxyurea *in vivo*, and then separated into cytoplasmic and nuclear extracts. These extracts were analyzed by western blotting to visualize the movements and expression amounts of Centrin2, XPC, and hHR23B.

Materials & Methods

Cell Culture

Two cell lines were used in this experiment MDA MB 231 and MCF-7. MDA MB 231 is a human breast cancer cell line with a high metastases rate. This cell line expresses low levels of Centrin2 does not express XPC, and has a mutated p53 gene. MCF-7 is also a human breast cancer, but it has a low metastases rate, expresses levels of Centrin2 and XPC that are comparable to non carcinomic human breast cells, and contains wild type p53 gene.

For regular culturing of the cells, 1.0 x 10^6 cells of each line were added onto two 100mm plates and grown in culture medium. The cells were incubated in a 5% CO_2 environment, at 37C° for seven days, and the medium was changed on the third and the sixth days. After seven days the medium was aspirated and the cells were washed twice for three minutes with warm PBS. The cells were then treated with 2ml of Versene Trypsin and incubated for five minutes. After incubation, the Trypsin was deactivated by adding 3mls of medium. The cells were then detached by gentle agitation, and examined to verify disassociation. The cells and medium were then pipetted out of the 100mm plate and put into a 15ml conical tube and centrifuged at 200 x *g* for 5 minutes. The supernatant was drawn off and the cells were re-suspended in 10 milliliters of fresh medium. The cells were counted using a hemocytometer and 1 x 10^6 cells were reseeded onto 8 new 100mm plates. The remaining cells were kept for future experiments.

Immediately following reseeding, four of the plates were treated with 2μM hydroxyurea (HU) for 48 hours, and four were kept as controls and allowed to incubate for the same time as the other condition.. After the 48 hour treatment, the cells were harvested and counted using the same method as above. The same numbers of cells were used for each experimental group.

Cellular Fractionation

Cells were washed twice with warm PBS. Two milliliters of Versene Trypsin was added, and the cells were incubated at 37°C for five minutes or until they detached from the plate. Four milliliters of cell medium was added to inhibit the trypsin, and the cells were then transferred to a 15ml conical tube and spun for five minutes at 200 x *g*. The medium was aspirated from the pellet, and five milliliters of ice-cold hypotonic buffer was added. The buffer and pellet was pipetted up and down ten times to mix, and then the cells were spun at 200 x *g* for five minutes.

After centrifugation, the cells were resuspended in one milliliter of hypotonic buffer and placed on ice for 10 min. The cells were then vortexed to resuspend and aid in rupturing them. Five hundred microliters of lysate was aliquoted into a 1ml centrifuge tube. This constituted the whole cell lysate. The remaining 500 μL of lysate was treated with 50μl of 3% acetic acid (10μl acetic acid/100μl hypotonic buffer) vortexed for 10 seconds, and then 50μl of 10% NP-40, buffer (10μl NP-40/100μl hypotonic buffer) was added. The cells were vortexed again for 10 seconds and visualized under a microscope. These lysates

were then centrifuged at 2500 rpm for 10 minutes at 4°C. The pellet constituted the nuclear fraction and the supernatant was used as the cytoplasmic fraction.

Western Blotting

After the whole cell, cytoplasmic and nuclear fractions were prepared, 70µl of Laemmli buffer was added to each, and they were vortexed for 30 seconds. All samples were then sonicated at 40 cycles per minute for 3 x 5 seconds and heated at 80°C for 4 minutes. All samples and controls were then loaded onto a 4 to 15% gradient of polyacrylamide gel and run at 200 volts for 50 minutes. The proteins from the gels were transferred onto a sheet of PVDF paper by electrotransfer (200mA, 60 minutes). Blots were then fixed in gluteraldahyde, blocked in milk overnight, and incubated with the appropriate antibody. The following primary antibodies were used: mouse monoclonal anti-Superoxide Dismutase (SOD), mouse monoclonal anti-Centrin, XPC polyclonal, anti-Phospho-histone H3, and anti-Phospho Gamma h2AX. The blots were washed extensively and then incubated with the appropriate secondary antibody fused to horseradish peroxidase and then developed using a chemiluminescent substrate.

Results

An efficient method of cellular fractionation was used in these experiments in which a hypotonic buffer was added to the cells to cause them to swell, after which the cells were ruptured by vortexing. This method provided a clean separation of the nuclear and cytoplasmic portions but did not damage the nuclear extracts. In figure 1-A a bright field microscopy image was taken of the cells in the hypotonic buffer before they ruptured. Figure 1-B shows another bright field microscopy image taken of nuclear portions of the cells after they had been vortexed and lysed. It is clear from the images that the nuclei are completely free from the cytoplasm.

In order to determine whether cellular fractions (ie cytoplasm and nuclei) were well separated from each other, western blots were prepared. Figure 2-A is a western blot of superoxide dismutase (SOD), a cytoplasmic protein that is not found in the nucleus of a cell. The western blot clearly shows that there is no cytoplasmic contamination in the nuclear extract, since no SOD can be detected in these extracts. To be sure there was no nuclear extract in the cytoplasmic fractions; a separate western blot, shown in Figure 2-B, was prepared for phospho histone H3. This protein is present when histones become phosphorylated, and has been demonstrated to be positive marker indicating DNA damage (Thirit, C., & Hays, J, 2005).

In both cell lines, DNA damage was induced by adding hydroxyurea (HU) to the cells *in vivo*. The concentrations of HU used in the experiments presented in this paper were tested for the ability to induce DNA damage by preparing cellular fractions and analyzing them by western blotting. Figure 3 shows a western blot of whole cell, cytoplasmic, and nuclear extracts from MCF-7 and MBA MD 231 cells. DNA damage results in an accumulation of phospho gamma H2AX, a known histone modification that correlates with DNA damage. The histone becomes phosphorylated when the cell detects, and attempts to fix double and single strand DNA breaks. The strong bands in the whole cell (W) and nuclear (N) fractions indicate that DNA damage has taken place and that the machinery necessary for DNA repair, has

been recruited. In the MCF-7 cells the bands of H2AX are much darker then in the 231 cells indicating more rigorous repair is taking place.

Figure 4-A shows a western blot of Centrin2 levels detected in MCF-7 and 321 cells treated with HU using an anti-Centrin2 mouse monoclonal antibody. It demonstrates that intracellular Centrin2 amounts increase upon DNA damage in both MCF-7 cells as well as MDA 231 cells; however, the increase is smaller in the MDA MB 231 cells. In the MCF-7 cells, Centrin2 is present in both the cytoplasm as well as in the nucleus after DNA damage, and the Centrin2 levels in the cytoplasm diminish and the levels in the nuclear fraction increases. In Figure 4-B, MCF-7 cells were western blotted for Centrin2, hHR23B, and XPC. The blots indicate an increase in intracellular amounts of all three proteins following HU treatment. Furthermore, HU treatment resulted in hHR23B increasing in total cellular volume, and it moved into the nucleus. XPC increased only in the nucleus of the cells.

Discussion:

The experiments presented in this paper demonstrate that XPC, Centrin2, and hRAD23B were all present in the nucleus following DNA damage by hydroxyurea and that the intracellular levels of all three proteins increased. These results confirm previous immunofluorescence microscopy data that showed DNA damage increased Centrin2 and XPC expression. Centrin2 translocated to the nucleus following DNA damage and that XPC is only present in the nucleus of cells and not in the cytoplasm before or after DNA damage. Based on these data it is not believed that XPC leaves the nucleus during GGNER in order to bind to Centrin2 or hRAD23. The previous study however did not investigate hHR23b's expression or movement following DNA damage; the results presented here clearly demonstrate that hHR23b exhibits a similar response in comparison to Centrin2 and XPC. These results are reasonable, since hHR23b is known to be a checkpoint protein. Immunofluorescence imaging still needs to be performed to confirm hHR23's nuclear translocation.

The mechanistic details by which XPC, Centrin2, and hHR23 translocate into the nucleus remain to be established. Perhaps one of the most interesting questions that remain to be investigated is whether or not XPC, Centrin2, and hHR23b physically associate together during DNA damage. The proteins may migrate to the nucleus independently of each other or as a multiprotein complex in response to DNA damage. A possible model is that as the histones become phosphorylated and stop the cell cycle in the G1 phase hHR23B moves into the nucleus in preparation for the S phase. It is known that hHR23B binds to the S5a subunit changing its conformation from closed to open and allowing it to bind to, and hold on to ubiquitylated substrates very tightly. This hHR23B complex might then bind preferentially to ubiquitylated XPC, but thus far the true mechanism is unknown.

Furthermore, a time course analysis should be performed to verify DNA repair has been started and finished and that Centrin2 levels return to pre-damage levels. Future experiments using co-immunoprecipitation could provide more information as to how XPC, Centrin2, and hHR23B bind and work together during GGNER.

Of the three proteins investigated in this study, Centrin2 may be the most important for a number of reasons. It has been reported that cells which express low levels of Centrin2 not only tend to fail cytokinesis, but they also tend to form multi polar spindle bodies. In these cells the centrosomes which normally migrate to opposite ends of the cell and provide an anchoring platform during mitosis, for some reason multiply producing three or more spindle poles. Although these bodies are not known to be a cause of cancer in and of themselves, they are found in an extremely high percentage of cancers. It has been suggested that, (Salisbury, 2004), (D'Assoro, 2002) & (Lingle,1999) these multi polar spindle bodies potentially damage the chromosomes during the cell cycle, since the chromosomes will no longer be able to segregate normally. Abnormal chromosome segregation may exacerbate the proliferative and metastatic behavior of cancer cells, since chromosomal DNA would be unequally divided between daughter cells. If accumulation of multi polar spindle bodies is caused by low levels of Centrin2, finding a way to modulate the levels of Centrin2 may provide a way of reducing the risk of developing more aggressive cancerous phenotypes.

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Figure Legends

Figure 1:

A: Swollen Cells in Hypotonic Buffer

This is a light field microscopy image of MCF-7 cells. Hypotonic buffer has been applied to the cells causing them to swell.

B: Nuclear Extract

This is a light field microscopy image of MCF-7 cells. After the cells have been vortexed the nucleus separates efficiently from the rest of the cell.

Figure 2:

A: Cell Fractionation with superoxide dismutase (SOD).

MCF-7 cells were treated with HU for 48 hours and western blots prepared for SOD which is a cytoplasmic protein. This is used to verity the quality of the fractionation. Note that there is no SOD present in the nuclear extracts.

B: Anti-Phospho histone H3

MCF-7 cells were treated with HU for 48 hours and western blots prepared for Anti phospho histone H3 which is a nuclear marker. This verifies the quality of the nuclear fraction. Note that there are no nuclear extracts present in the cytoplasmic extracts.

Figure3:

A: DNA damage indicated by Phospho Gamma H2AX

This is a western blot of phospho gamma histone H2AX. This shows where DNA damage has taken place, and as stated earlier it also indicates that DNA repair is taking place. In prior unpublished research, the Salisbury Laboratory prepared an immunofluroencence image of H2AX, and this western blot confirms those earlier data.

Figure 4:

A: Centrin2 levels increase intracellularly in MCF-7 and 231 cells Upon DNA damage

This is a western blot of MCF-7 cells and MDA-MB 231 cells prepared for Centrin2 following DNA damage caused by hydroxiurea. The levels of Centrin2 increase in the MCF-7 cells after DNA damage has been induced. Also the Centrin2 moves out of the cytoplasm and into the nucleus.

B: Centrin2, XPC and hHR23B all increase intracellularly upon DNA damage

Mosaic figure of western blotted proteins isolated from MCF-7 cells and treated with hydroxyurea causing DNA damage. The levels of XPC, hHR23B, and Centrin2 all increase. XPC increased only in the nuclear extract, and hHR23B and Centrin2 both increased and moved into the nucleus.

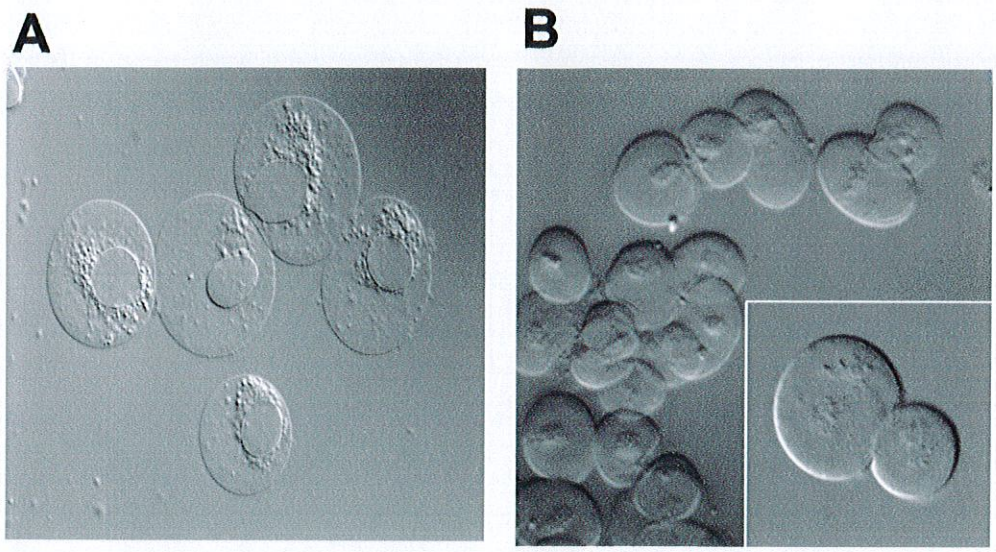

Figure 1

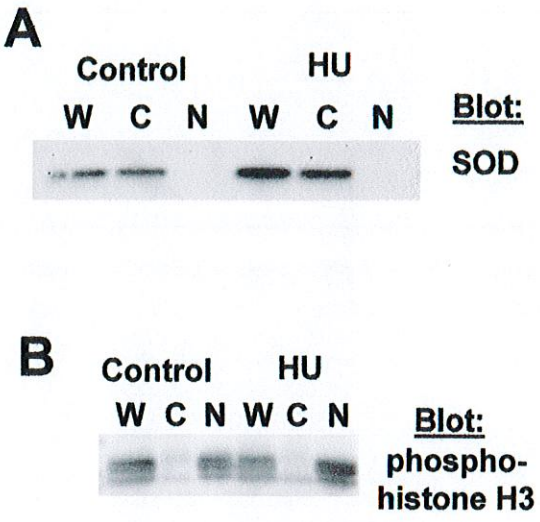
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Figure 2

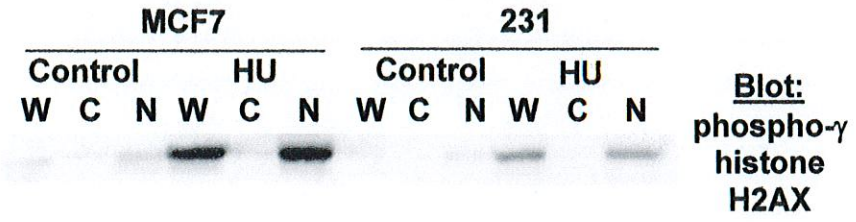
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Figure 3

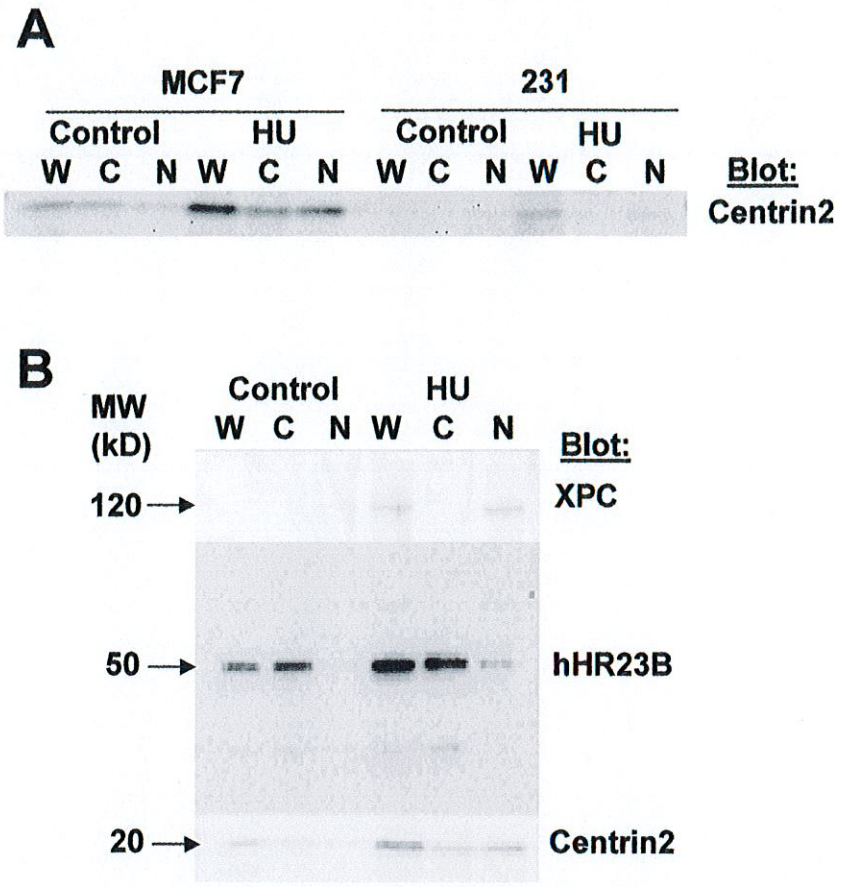
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Figure 4