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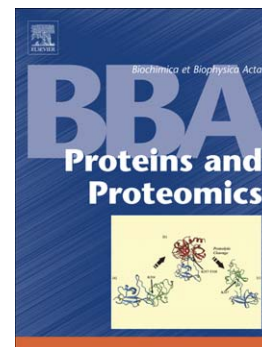
Mass Spectrometry analysis of the oxidation states of the pro-oncogenic protein Anterior Gradient-2 reveals covalent dimerization via an intermolecular disulphide bond.

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1

SUMMARY

Anterior Gradient-2 (AGR2) is a component of a pro-oncogenic signalling pathway that can promote p53 inhibition, metastatic cell migration, limb regeneration, and cancer drug-resistance. AGR2 is in the protein-disulfide isomerase superfamily containing a single cysteine (Cys-81) that forms covalent adducts with its client proteins. We have found that mutation of Cysteine-81 attenuates its biochemical activity in its sequence-specific peptide docking function, reduces binding to Reptin, and reduces its stability in cells. As such, we evaluated how chemical oxidation of its cysteine affects its biochemical properties. Recombinant AGR2 spontaneously forms covalent dimers in the absence of reductant whilst DTT promotes dimer to monomer conversion. Mutation of Cysteine-81 to alanine prevents peroxide catalyzed dimerization of AGR2 *in vitro*, suggesting a reactive cysteine is central to covalent dimer formation. Both biochemical assays and ESI mass spectrometry were used to demonstrate that low levels of a chemical oxidant promote an intermolecular disulfide bond through formation of a labile sulfenic acid intermediate. However, higher levels of oxidant promote sulfinic or sulfonic acid formation thus preventing covalent dimerization of AGR2. These data together identify the single cysteine of AGR2 as an oxidant responsive moiety that regulates its propensity for oxidation and its monomeric-dimeric state. This has implications for redox regulation of the pro-oncogenic functions of AGR2 protein in cancer cells.

INTRODUCTION

Oesophageal adenocarcinoma is thought to develop from pre-malignant lesions termed Barrett's oesophagus [1]. Selection pressures are known to drive p53 gene mutation early in oesophageal cancer progression [2]. Whole genome sequencing has determined that selection pressures for p53 gene mutations are remarkably confined to a highly specific stage, that being Barrett's tissue associated with high-grade dysplasia [3]. Barrett's epithelium forms a relatively unique microenvironment for the identification of stress activated p53 modifiers might place selection pressures on the survival of cells with either p53 gene mutation or in maintenance of the wt-p53 alleles [4-6]. Using a proteomics approach in Barrett's epithelium to identify such possible p53 modifiers, a protein named Anterior Gradient-2 (AGR2) was identified and validated as an abundant and potent inhibitor of p53-dependent transcription [7, 8]. AGR2 remains upregulated in a large proportion of oesophageal adenocarcinomas [9]. The recent generation of an AGR2 isogenic cell panel highlighted p53 pathway suppression as the dominant effect of enhanced AGR2 protein-dependent remodelling of the proteome [10]. Thus, AGR2 pathway function forms a core proteomic landscape whose study might shed light on oesophageal adenocarcinoma development as well as p53 pathway silencing.

AGR2 was originally identified as a secretory protein that is highly expressed in Xenopus eggs [11]. Apart from its function as a p53 inhibitor [8], subsequent studies have shown a significant role for AGR2 in a range of biological pathways including cell migration, cellular transformation, metastasis [12] [9], and limb regeneration in vertebrates [13]. Clinical studies have also implicated the protein in inflammatory bowel disease [14], hormone-dependent breast cancers [15] [16], and in predicting poor prognosis in prostate cancers [17]. The molecular mechanisms underlying these wide-ranging biological pathways trigged by AGR2 are still not completely defined and as the AGR2 gene is confined to vertebrates, the AGR2 gene pathway cannot be dissected using powerful genetic systems like yeast, flies, or worms [18]. However, emerging functions in the AGR2 pathway focus on (i) identifying its client proteins as it mediates protein folding in the endoplasmic reticulum [19] and as it stimulates receptor maturation [20] [21]; (ii) understanding its function in the endoplasmic reticulum in response to unfolded protein responses [22], (iii) defining the nature of its monomer-dimer equilibrium in tis chaperone cycle [23]; and (iv) understanding the significance of its highly specific peptide docking function which is relatively unique for a molecular chaperone [24] [25].

Endoplasmic reticulum localized molecular chaperones like AGR2 have specific roles that enable the folding, trafficking, and assembly of complex cysteine-rich transmembrane

3

receptors with unique protein folding requirements. Reduced glutathione and its oxidized counterpart comprise the dominant redox buffer in eukaryotes [26]. A relatively high pro-oxidizing environment in the endoplasmic reticulum is thought to facilitate the iterative cycles of enzymatic cysteine reduction and oxidation on cysteine-rich client receptors destined for the secretory system and transmembrane destinations [27]. Classic thioredoxins have a conserved thioredoxin fold comprised of the CxxC motif that mediates covalent bond formation with cysteine containing client proteins followed by resolution through cycles of reduction-oxidation [28]. AGR2 by contrast is part of the thioredoxin superfamily that contain CxxS motifs and which lack the ability to exploit a two cysteine redox system that classically mediates client protein oxidation and reduction cycles [29]. As such, AGR2 single cysteine oxidation and reduction could form an important rate-limiting step in its reaction cycle with implications for client protein maturation in human diseases like cancer. In this report, we detail the effects of single cysteine mutation on the known core biochemical function of AGR2 and also the nature of AGR2 cysteine oxidation on its oligomerization state using mass spectrometry (MS). We demonstrate first that cysteine mutation can alter its specific activity in protein-interaction assays. Secondly, we demonstrate that low levels of cysteine oxidation can induce covalent dimerization through an unstable sulfenic acid intermediate. Finally, we show that higher levels of cysteine oxidant yield a sulfinic or sulfonic acid product that traps AGR2 in the monomeric and oxidized state. These assays will facilitate future dissection of the reaction mechanism of AGR2 as it mediates protein folding of cysteine rich client proteins. This may reveal how its oxidation state in the endoplasmic reticulum might effects its specific activity as an oncogenic chaperone in the development and maintenance of the transformed phenotype.

EXPERIMENTAL PROCEDURES

General

All reagents were purchased from Sigma unless otherwise stated.

In Vivo Cross-linking of proteins in MCF7 Breast Cancer Cells

MCF7 breast cancer cells were cultured in DMEM supplemented with 10% FCS in a humidified incubator in 5% CO_2 at 37ºC. For cross-linking, cells were grown to ~90% confluence before exposure to DSS (Pierce 21555) or EGS (Pierce 21565). DSS or EGS was dissolve in 100% DMSO to a concentration of 100mM before being further diluted to the appropriate concentration in growth media and added to cells. Cells were incubated for a further 1 hour at 37ºC before being harvested into PBS and lysed into the following buffer: 150mM NaCl, 50mM Tris HCl pH 8.0, 50mM NaF, 5mM EDTA, 1% NP-40 plus 1:100

4

phosphatase inhibitor cocktail (Sigma P5726) and 1:100 protease inhibitor cocktail (Sigma P8340). AGR2 containing cross-linked complexes were detected by Western blot analysis using a polyclonal antibody to AGR2 raised in rabbit (Moravian Biotechnology Ltd., Brno Czech Republic).

Site Directed Mutagenesis of AGR2 at Cysteine 81

Human AGR2 was cloned into Invitrogen's Gateway expression vectors pDEST17 for bacterial expression or pDEST12.1 for mammalian expression [8]. Site-directed mutagenesis was then carried out on these constructs using Stratagene's QuickChange® Site-Directed Mutagenesis Kit as per manual. Mutagenic oligonucleotide primers used for C81A were as follows; site of mutation underlined: forward: 5'-GATTATTCATCACTTGGATGAGGCCCCACACAGTC,reverse:5'-GACTGTGTGGGGCCTCATCCAAGTGATGAATAATC. Mutagenic oligonucleotide primers used for C81S were as follows; site of mutation underlined: forward: 5'-GATTATTCATCACTTGGATGAGTCTCCACACAGTC,reverse:5'-GACTGTGTGGAGACTCATCCAAGTGATGAATAATC.

In vivo Cross-linking of Transfected wt and C81A AGR2

H1299 lung carcinomas cells were cultured in RPMI supplemented with 10% FCS in a humidified incubator in 5% CO_2 at 37ºC. One day prior to transfection cells were plated into 6 well plates (2ml media) so that they would be 90-95% confluent the following day. Plasmid DNA (1μg) encoding either wt or C81A AGR2 and Lipofectamine™ 2000 (10 μl) were added to two separate volumes of RPMI w/o serum (250 μl) and both were incubated for 5 mins at RT. After this time the two mixtures were combined, gently mixed and incubated at RT for a further 20 mins at RT before addition to cells. Cells were incubated for 24 hours before cross-linking was carried out as described above using 1mM DSS.

In vitro Oxidation of Recombinant AGR2 to Form Homodimer

His-tagged AGR2 purified as described previously [8] was incubated in various concentrations of H_2O_2 for 4 hours at RT. After this time 4 μg of each reaction was analysed by SDS-PAGE carried out w/o the addition of DTT to sample loading buffer. The resulting gel was stained with Coomassie Blue. For mass spectrometry, AGR2 was concentrated to 50 μM in ammonium acetate (100 mM, pH 7.2) before reaction with H_2O_2. Oxidation was allowed to proceed for various times before the addition of 4-fold H_2O:MeOH:HCOOH (50:45:5) (v/v) quenched the reaction. Reptin was purified as follows; the gene was cloned in frame with an N-terminal precision protease sequence into pDEST15 vector,

containing glutathione S-transferase N-terminal tag, expressed into BL21 (DE3) competent E. coli cells and grown O/N. The cells were subcultured and induced with 1M IPTG after OD 0.4 had been reached. After 3 hours the cells were pelleted and incubated with lysis buffer (10% sucrose, 50mM Tris (pH 8), 150mM NaCl, 3mg Lysozyme, 0.5% NP40, 5mM DTT, 1mM Benzamidine, 20μg/ml leupeptin, 1μg/ml aprotinin, 2μg/ml pepstatin, 10μg/ml soybean trypsin inhibitor, 1mM EDTA) for 45 minutes before a 1-minute incubation at 37°C and sonication on ice. The lysates were then centrifuged at 4000rpm for 15 minutes and the supernatant added to glutathione sepharose 4B beads and incubated for 2 hours at 4°C. The beads were washed extensively using wash buffer (10 x with 20mM HEPES (pH 7.5), 1mM DTT, 10% glycerol, 150mM NaCl, ten times with 20mM HEPES (pH 7.5), 1mM DTT, 10% glycerol, 1.0 M NaCl, and two times with 20mM HEPES (pH 7.5), 1mM DTT, 10% glycerol, 150mM NaCl) before elution buffer (25mM HEPES (pH 7.5), 1mM DTT, 10% glycerol, 150mM NaCl) containing GST-tagged PreScission protease. The mixture was incubated O/N at 4°C and then eluted Reptin was measured for purity using an SDS-Coomassie blue stained gel and concentration by Bradford reagent (Sigma).

Modification of AGR2 with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NDB-Cl). A solution containing 50 μM AGR2 in ammonium acetate (100 mM, pH 7.2) was treated with 1 mM H_2O_2 in the presence of 4 mM NDB-Cl. The reaction was allowed to proceed for 45 minutes in the dark. H_2O:MeOH:HCOOH (v/v/v 50:45:5) was then added to quench the reaction and leave the sample at a final concentration of 10 μM for MS analysis. A reaction without H_2O_2 was used as a control.

Protein mass spectrometry. Before mass spectrometry analysis each sample was denatured with H_2O:MeOH:HCOOH (v/v/v 50:48:2), resulting in a protein concentration of approximately 10 μM. Mass spectrometry data was acquired on an apex ultra Qh-FT-ICR mass spectrometer equipped with a 12 Tesla magnet and an electrospray ion source (Bruker Daltonics, Billerica, MA). Nano-ESI was performed using a TriVersa Nanomate (Advion BioSciences, Ithaca, NY) running in infusion mode. Desolvated ions were transmitted to a 6 cm Infinity cell® penning trap. Trapped ions were excited (frequency chirp 48-500 kHZ at 100 steps of 25μs) and detected between *m/z* 600 and 3000 for 0.5 s to yield a broadband 512K- or 1 Mword time-domain data. Each spectrum was the sum of 32 mass analyses. The mass spectra were externally calibrated using ES tuning mix (Agilent) and analysed using

DataAnalysis software (Bruker Daltonics). Isotope distributions of specific charge states were predicted using IsotopePattern software (Bruker Daltonics) from theoretical empirical formulae. These were overlaid upon the recorded experimental data as scatter plots, with the theoretical apex of each isotope peak designated by a circle.

<u>Peptide ELISA to Measure Affinity of wt and mutant AGR2 to Peptide Aptamers and Reptin</u>

For measuring AGR2 binding to Reptin; the wells of an ELISA plate were coated with 50 μL of recombinant Reptin (10 μg/mL) overnight at 4ºC in 0.1M Sodium Carbonate buffer (pH 9.0). The following day the wells were washed six times with 200 μL of PBST and blocked with 200 μL of 3% BSA in PBST for 1 h. A dilution series of either wt or C81A/K95R recombinant His-tagged AGR2 was prepared and added to the wells for 1 hr at room temperature. The wells were then washed six times with PBST and probed for the presence of AGR2 using polyclonal AGR2 antibody diluted 1:2000 in blocking buffer. The reaction was detected using swine anti-rabbit HRP monoclonal antibody diluted 1:2000, developed by ECL, and read using Fluoroskan Ascent FL. For measuring AGR2 binding to synthetic peptides; the wells of an ELISA plate were coated with 50 μL of streptavidin (2 μg/mL in H_2O) overnight at 37 ºC. The following day the wells were washed four times with 200 μL of PBST, 50 μL of synthetic peptides (D8, E7, or F3; 0.1 mg/mL) was added for 1 hr at room temperature, and the wells were washed six times with 200 μL of PBST and blocked with 200 μL of 3% BSA in PBST for 1 h. The sequences of D8, E7, and F3 are, HLPTTIYYGPPG, YPWHHSWTHTTL, and NTSSMPIFSTNR, respectively. A dilution series of either wt or C81A/K95R recombinant His-tagged AGR2 was prepared and added to the wells for 1 hr at room temperature. The wells were then washed six times with PBST and probed for the presence of AGR2 using polyclonal AGR2 antibody diluted 1:2000 in blocking buffer. The reaction was detected using swine anti-rabbit HRP monoclonal antibody diluted 1:2000, developed by ECL, and read using Fluoroskan Ascent FL.

<u>Accumulation of Transfected AGR2 after MG132 Treatment</u>

HCT116 colon cancer cells (wt-p53 and isogenic p53-null cells) were cultured in McCoy's 5A media supplemented with 10% FCS in a humidified incubator in 5% CO_2 at 37ºC. One day prior to transfection cells were plated into 6 well plates (2ml media) so that they would be 90-95% confluent the following day. Transfections were carried out using Attractene transfection reagent (Qiagene). Either wt or C81A AGR2 or empty vector control (0.6µg) and Attractene (2.25µl) were added to media w/o serum to a final volume of 500 µl and incubated at RT for 10 mins before being added to the cells and incubated for 24 hours. After this time media was removed and replaced with fresh media containing either 0 or 10 µM MG132

(Calbiochem) and incubated for a further 5 hours. Cells were then harvested, lysed and AGR2 levels were measured by Western blot as described above.

RESULTS

Cys81 mutation affects the biochemical and cellular stability of AGR2 protein.

Dimer to monomer stability can regulate the specific activity of AGR2 in protein-binding assays; most notably, deletion of its N-terminal dimer instability domain can increase its activity [30]. We first aimed to develop an understanding of whether mutation of Cys81 affects the AGR2 dimer-monomer equilibrium of AGR2. The use of amino acid-reactive chemicals were initially used as tools to define dimerization determinants. The cell membrane permeable cross linker DSS (but not EGS) induced a predominant dimerization of AGR2 in cells (Figure 1A). We observed that this DSS-mediated chemical oxidation occurs equally well in wild-type AGR2 or the AGR2[C81A] cysteine mutant (Figure 1B) indicating that DSS mediated dimerization in cells does not proceed through cysteine oxidation. This is consistent with previous data showing that *in vitro*, the DSS reactive cross-link that stabilizes AGR2 dimerization maps to K95[30]. These data also indicate that mutating Cys81 does not alter the fixed distance between Lys95 residues in the AGR2 dimer that facilitate DSS-dependent cross-linking. In addition, we noticed that the AGR2[C81A] mutant protein exhibited reduced steady state levels than wt-AGR2 (Figure 1B, lanes). As such we evaluated whether the instability of the AGR2[C81A] mutant protein was proteasome dependent; indeed the lower steady state levels of the AGR2[C81A] mutant protein were elevated by the proteasome inhibitor MG132 (Figure 1C). These data suggests that *in vivo* modification of Cys81 of AGR2 could alter its steady state levels.

Next we evaluated whether there are any differences in the specific activity of AGR2 as a function of Cys81 mutation. First, we observed that the AGR2[C81A] mutant protein is defective in binding to the chaperone protein Reptin (Figure 1D). Similar loss of binding to Reptin was observed when using the AGR2[K95R] mutant protein (Figure 1D). The biochemical activity of the AGR2[C81A] mutant protein towards its consensus peptide-docking site was also reduced compared to wt-AGR2 (Figure 1E). As such, we focused in this study on measuring whether the more physiologically relevant cysteine oxidation can impact on the dimeric subunit structure of AGR2.

Oxidation-dependent dimerization of AGR2 dependent upon Cys81

To evaluate the effects of cysteine oxidation on AGR2 dimer stability, we purified his-tagged AGR2 (larger mass than untagged AGR2 due to the amino acid tag) in the absence of reducing agent (normally used during purification) that resulted in the accumulation of a

larger molecular mass species of approximately twice the mass of his-tagged AGR2 (Figure 2A, lane 1). The incubation of the sample with the reducing agent DTT resulted in collapse of the ~48 kDa species (Figure 2A, lane 2 vs 1) suggesting a cysteine-dependent covalent dimer was formed under pro-oxidizing conditions. AGR2 protein has one reactive cysteine at codon 81 (Figure 2B) and mutation of this amino acid to alanine prevented the spontaneous oxidation-dependent dimerization (Figure 2C, lane 3 vs 1).

In order to determine whether the covalent dimeric form of AGR2 can form through such an oxidation, we needed to set up an *in vitro* oxidation system that can be used to monitor quantitatively covalent dimerization of reduced and monomeric AGR2 protein. A titration *in vitro* of H_2O_2 into a solution with reduced AGR2 protein results in the appearance of dimeric AGR2 (Figure 3A, lane 2 vs 1), whilst at concentrations greater than 200 μM the dimerization was attenuated (Figure 3A, lane 5-9 vs lane 2-4). A titration of H_2O_2 at reduced concentrations demonstrates that dimerization is promoted from a concentration of 5-100 μM (Figure 3B, lanes 5-8 vs lanes 1). The use of AGR2[C81S] and AGR2[C81A] mutant proteins confirmed that the peroxide induced dimerization was dependent upon Cys81 (Figure 4A-C). These data provide a dynamic assay that can be used to chemically oxidize reduced AGR2 to either promote dimerization at low H_2O_2 concentrations or to prevent AGR2 dimerization at elevated concentrations of H_2O_2. This suppression of AGR2 oxidation at elevated concentrations of H_2O_2 suggests a mechanism whereby oxidation levels can regulate the conversion of AGR2 monomer to dimer. This might prove in future to have effects in cells on the specific activity of AGR2 in pro-oxidizing environment of the endoplasmic reticulum.

Oxidation-dependent dimerization of AGR2 through direct Cys81 modification

We next set out to determine whether, indeed, chemical evidence can be obtained for direct Cys81 oxidation through a Cys81-Cys81 cross link but also to explain why higher concentrations of oxidant prevents AGR2 covalent dimerization. ESI mass spectrometry was used to determine whether such a reaction mechanism could be developed by analysing changes in molecular mass of full-length AGR2 as a function of increasing oxidant and therefore whether evidence could be found for dimerization through a disulfide bond or through a distinct amino acid.

The AGR2 protein used has an N-terminal histidine-tag (Figure 5A). After rapid replacement of reduced his-tagged AGR2 protein in ammonium acetate buffer a $H_2O/MeOH/HCOOH$ electrospray make-up solvent was added in order to denature the protein before direct infusion MS analysis. The protein showed a charge distribution from [M+12H][12+] to

[M+24H]$^{24+}$ (Figure 5B and Figure 6) with an nominal average mass of 21,041 Daltons for the cysteine-reduced protein. Analysis of the isotope distribution of the [M+20H]$^{20+}$ charge state is consistent with the theoretical isotope distribution of monomeric AGR2 containing one reduced cysteine residue [C$_{944}$H$_{1506}$N$_{258}$O$_{277}$S$_{5}$]$^{20+}$ (Figure 5B, insert). Gel filtration confirmed that AGR2 protein in the reduced state does not form a stable homodimer (data not shown; [30]). A titration of AGR2 with the oxidant H$_2$O$_2$ was performed and monitored by mass spectrometry, which resulted in the appearance of a second species in the mass spectrum. This species displayed a charge state distribution from [M+23H]$^{23+}$ to [M+31H]$^{31+}$, and a nominal average mass of 42079 Da; the signals of these ions were elevated in response to increasing oxidant (Figure 6). This observation is consistent with the formation of a dimeric AGR2 species, as suggested using gel electrophoresis (Figure 3 and 4). Indeed, an expanded spectrum of the ion at [M+27H]$^{27+}$ charge state is consistent with the theoretical isotope distribution of an AGR2 dimer containing one intermolecular disulfide bond [C$_{1888}$H$_{2997}$N$_{516}$O$_{554}$S$_{10}$]$^{27+}$(Figure 7A and B). These data together indicate that the reactive cysteine plays a direct role in covalent dimer formation (Figure 7C).

Interestingly, the additions of higher levels of oxidant prevent dimer formation (Figure 4). To investigate why dimerization might be suppressed at higher oxidant concentrations, we also analysed this reaction mechanism using mass spectrometry. Expanded analysis of the ions isolated with a [M+15H]$^{15+}$ charge state at higher oxidant concentrations resulted in the loss of the dimer and accumulation of ions with monomeric mass consistent with the addition of two oxygen atoms [C$_{944}$H$_{1501}$N$_{258}$O$_{279}$S$_{5}$]$^{15+}$, suggesting oxidation of the cysteine residue within AGR2 to the sulfinic acid, Cys-SOOH (Figure 8A and B). The highest concentrations of oxidant also yielded a species with three additional oxygen atoms, [C$_{944}$H$_{1501}$N$_{258}$O$_{280}$S$_{5}$]$^{15+}$, consistent with formation of sulfonic acid, Cys-SO$_3$H (Figure 8A).

It is clear from the above results that, within AGR2, Cys-81 is susceptible to oxidation by peroxide. We have demonstrated that with excess peroxide treatment Cys-81 is readily oxidised to a sulfinic acid. However, under mild peroxide condition (< 200 μM), oxidation favours disulfide bond formation – resulting in an AGR2 dimer covalently coupled via an intermolecular disulfide bond. Both H$_2$O$_2$ mediated disulfide bond formation and over-oxidation of cysteine to sulfinic acid are thought to proceed through a labile sulfenic acid intermediate (Cys-S-OH). Species containing this oxidation state of cysteine are generally highly reactive and unstable to the ESI process making them unsuitable for MS analysis. However, it is known that the electrophilic agent NBD-Cl reacts specifically with both Cys-SH and Cys-S-OH to produce Cys-S-NDB adducts and Cys-S(O)-NDB adducts respectively, both with ∆mass +163 Da (Figure 9A) [31]. Crucially, upon reaction with NBD-Cl, the oxygen

atom from a sulfenic acid is retained within the product; producing a mass label that is stable to ESI-MS. Reduced or oxidized AGR2 protein was incubated with NBD-Cl prior to infusion for analysis using mass spectrometry. Under these conditions, the protein with charge state of $[M+18H]^{18+}$ had an increase in mass of 162.963 Da (Figure 9B) which is consistent with a single NBD adduct. The oxidized AGR2 mass at this charge state was elevated by 178.982 Da (Figure 9B) which is consistent with the trapping of an unstable sulfenic acid intermediate (Figure 9B). Together, these MS observations allow us propose a mechanism for peroxide-mediated dimer formation and propose a mechanism whereby excessive cysteine oxidation prevents dimer formation (summarized in Figure 10).

DISCUSSION

Our experiments aimed to define how the single cysteine in AGR2 impacts on its protein binding activity and oxidation-dependent dimerization. In this report, we first developed an *in vivo* assay that demonstrates that AGR2 appears to exist in a non-covalent dimeric state as defined by the ability of DSS to cross link AGR2 into a homo dimer (Figure 1A). This *in vivo* cross-link might occur through Lys95-Lys95 adducts (mapped *in vitro* [30]) and not through Cys81-Cys81 adducts (Figure 1B). However, the titration of the chemical oxidant peroxide suggested that AGR2 can assemble into a distinct dimeric structure that is mediated through a Cys81-Cys81 covalent bond (Figure 4). The discovery that AGR2 forms an oxidation responsive dimer opens the door to begin to analyse this fundamental property of AGR2 homodimer on its cell physiology. Mutation of the reactive cysteine to alanine reduced the specific activity of AGR2 in sequence-specific peptide-aptamer binding and in Reptin protein-binding assay (Figure 1D and E). This mutation also changes the *in vivo* stability of AGR2 (Figure 1C).

Mass spectrometry is a powerful tool for the characterisation of protein modifications by the monitoring molecular mass of the intact protein species; for example we have previously used this technique to define the stoichiometry of methylation and acetylation on native histones purified from HCT116 colon cancer cells [41] and to characterise reactive cysteine's on the p53 tumour suppressor [42]. In this current report, mass spectrometry was used to define the nature of the covalent modifications on AGR2 as a function of increasing oxidant concentration. Mass spectrometry has also been previously used to define the mechanism of sulfonucleotide reduction by sulfonucleotide reductase [43]. We were able to distinguish between a two models that invoked a reaction mechanism involving the reduction of an inter-molecular disulfide in a dimeric structure or an intramolecular disulfide bond in a monomeric form of the enzyme prior to nucleophilic attack on sulfonucleotides to form an enzyme-thiosulfonate intermediate. Covalent dimerization via disulfide bond formation appears to be

11

a increasingly important physiological mechanism involved in regulating signalling proteins. The adhesion factor E-cadherin can also form a DTT-sensitive dimer with dimerization driving the degradation of the protein in cells [44]. By contrast, dimerization of AGR2 appears to have physiological significance since the monomeric mutant protein (C81A) was significantly de-stabilized through the proteasome pathway in HCT116 colon cancer cells (Figure 1C). In turn, changes in AGR2 steady state levels in cells might regulate some of the many biological functions of AGR2 including cell migration, transformation, or p53 inhibition. Specifically, since the dimer to monomer conversion in AGR2 is responsive to the redox state of the protein, this suggests that in a pro-oxidizing environment like that observed in cancer cells and/or in the endoplasmic reticulum, AGR2 oxidation states might yield diverse regulatory pools of the protein. This has implications for AGR2 binding to Reptin in the cytosol since the cysteine mutation in AGR2 severely impacts on Reptin Binding (Figure 1). Indeed, it is interesting that AGR2 is more able to inhibit p53 protein activity after cells have been subjected to a pro-oxidizing stress of UVC irradiation [8] or cisplatin [10]. Additionally, AGR2 is also able to mediate export of p53 from the nucleus into the cytoplasm better upon UVC irradiation [25]. UVC is a known pro-oxidizing stimulus in cells [45, 46] and these data allow a model to be developed that proposes AGR2 protein activity might be stimulated by stresses present in a pro-oxidizing environment revealing a potentially physiological role for dimer assembly in AGR2-mediated oncogenesis.

Lastly, a recent report has confirmed that AGR2 is a PDI that is essential for the production of the intestinal mucin MUC2, a cysteine-rich glycoprotein that forms the protective mucus gel lining the intestine [35]. This agrees with other reports suggesting that AGR2 plays a role in mucus secretion [36] (and European patent WO/2004/056858). This is a major function of goblet cells and producing this protective mucus layer can have an impact in inflammatory bowel disease through inhibition of bacterial entry into intestinal cells. The cysteine residue within the AGR2 thioredoxin-like domain was shown to form mixed disulfide bonds with cysteines in the N-terminus or C-terminus of MUC2 as the receptor is being processed [35]. Thus we would expect that irreversible oxidation of AGR2 would impact upon mucin secretion and/or that there exist chemical control mechanisms in the ER that minimize sulfinic or sulfonic acid formation. This might involve other CxxC containing PDIs that prevent irreversible oxidation of AGR2. It remains to be determined if AGR2 interacts with such CxxC containing PDIs. Such control of AGR2 might be important for regulating inflammatory bowel disease, as $AGR2^{-/-}$ mice are highly susceptible to chemically-induced acute colitis [35]. Further studies are required to precisely determine how dietary chemicals or intracellular oxidation of AGR2 impacts its ability to interact with cysteine-rich client proteins like MUC2.

12

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FIGURE LEGENDS

Figure 1. Mutation of AGR2 at Cys81 alters its properties. (*A). In vivo treatment of living cells with cell-membrane permeable homobifunctional N-hydroxysuccimide esters.* MCF7 cells grown in media with 10% FCS were incubated with increasing concentrations of the cell-membrane permeable cross-linkers DSS (lanes 2-5) or EGS (lanes 6-9) for 1 hour at 37°C. Cells were harvested, lysed using a Tris-HCl (pH 8.0) buffer containing 1% NP-40, lysates were separated by electrophoresis, and protein was immunoblotted to determine whether intermediary species of AGR2 could be detected under conditions where the majority of AGR2 was not cross-linked. Higher molecular mass adducts that react with the AGR2 antibody is highlighted by arrows. (B). *Mutation of AGR2 at Cysteine-81 does not prevent in vivo dimerization induced by DSS*. H1299 cells were transfected with expression vectors including; vector only control (lanes 1), encoding wt-AGR2 (lanes 2 and 3), or AGR2^{C81A} protein (lanes 4 and 5). Cells were incubated with DSS and lysates were immunoblotted to examine extent of AGR2 cross-linking. The arrows highlight monomeric and dimeric AGR2 protein. *(C). The C81A mutation in AGR2 sensitizes to proteasome-dependent degradation in vivo in HCT116 colorectal cancer cells.* Vectors expressing wt-AGR2 or AGR2^{C81A} were transfected into HCT116 cells (lanes 1-6) or isogenic p53-null HCT116 cells (lanes 7-12). Twenty fours hours later, cells were treated with the proteasome inhibitor MG132 (as indicated by +) and cells were harvested for immunoblotting: AGR2; p53; and Actin loading control. *(D and E). Effects of C81A mutation of AGR2 on its Reptin and peptide-binding activity.* (D) Fixed amounts of Reptin (500 ng/well) were absorbed onto ELISA wells or (E) fixed amounts of the indicated biotinylated peptides (D8 and E7 are AGR2 binding peptides and F3 is a non-specific control) were adsorbed onto the streptavidin solid phase. Next, wt-AGR2, AGR2^{K95R} or AGR2^{C81A} (C2A mutant) proteins were titrated to measure relative binding affinities of AGR2 towards (D) Reptin or (E) the indicated synthetic

peptides using anti-AGR2 antibody. The data are plotted as extent of AGR2 binding (RLU) as a function of AGR2 titration (μg).

Figure 2. A reactive cysteine mediates covalent dimerization of AGR2. (A). *In vitro oxidation of AGR2 promotes homodimerization.* Recombinant his-tagged AGR2 protein (100 ng) was incubated without reducing agent (DTT; lane 1) or with DTT for 1 hour at 37°C (lane 2) prior to electrophoresis. Samples were immunoblotted for examining changes in the denatured mass of AGR2 protein. (B). *Primary amino acid sequence of AGR2 with Cysteine 81 highlighted. (C). Mutation of Cyteine-81 prevents oxidation induced AGR2 dimerization.* Recombinant his-tagged wt- AGR2 protein (lanes 1, 2, 4 ,and 5) or AGR2[C81A] protein (lanes 3, 4, 7, and 8) were incubated without or with reducing agent (DTT), as indicated. Samples were immunoblotted for examining changes in the denatured mass of AGR2 protein.

Figure 3. Oxidant-induced dimerization of AGR2. AGR2 protein was incubated with the indicated concentrations of hydrogen peroxide ((A) 0-10 mM and (B) 0-500 μM]), reactions were quenched using SDS sample buffer (without DTT), samples were separated by electrophoresis, and then stained with Coomassie blue. The arrows indicate position of monomeric and dimeric AGR2.

Figure 4. A reactive cysteine mediates covalent peroxide-induced dimerization of AGR2. AGR2 protein (wild type, C81A or C81S) were incubated with the indicated concentrations of hydrogen peroxide (H_2O_2), reactions were quenched using SDS sample buffer (without DTT), samples were separated by electrophoresis, and then stained with Coomassie blue. The arrows indicate position of monomeric and dimeric AGR2

Figure 5. Intact Mass Spectrometry analysis of histidine-tagged AGR2. (A) Amino acid sequence of AGR2 with the histidine tag (underlined), the theoretical empirical formula (without N-terminal methionine residue) is highlighted. (B) AGR2 protein affinity purified form nickel-agarose column was buffer exchanged into 100 mM Ammonium Acetate (pH 7.2) and the protein concentration was adjusted to 0.5 mg/ml. Aliquots (10 μl) were mixed with 20 μl of a electrospray make-up solvent containing 45% methanol and 5% formic acid. Samples were directly infused and analysed by nESI MS. The data depicts the charge state distribution. Analysis of the isotope distribution of the $[M +20H]^{20+}$ charge state is highlighted, *insert*.

Figure 6. Effects of oxidant titration on the charge state distribution of AGR2 protein. AGR2 protein (50 μM) affinity purified form nickel-agarose column was buffer exchanged into

100 mM Ammonium Acetate (pH 7.2), incubated with the indicted concentrations of the oxidant H_2O_2 (final concentration 6.2 μM to 2.5 mM) for 20 minutes before the reaction was terminated using a 3x volume of electrospray make-up solvent. Samples were directly infused and analysed by nESI MS. Data depicts the mass-charge distribution (from $[M+20H]^{20+}$ to $[M+12H]^{12+}$) of AGR2 highlighted as a function of H_2O_2 concentration. Dose dependent formation of a dimeric species was observed with increasing oxidant concentration. Charge states corresponding the dimeric species are highlighted in red (from $[D+31H]^{31+}$ to $[D+23H]^{23+}$).
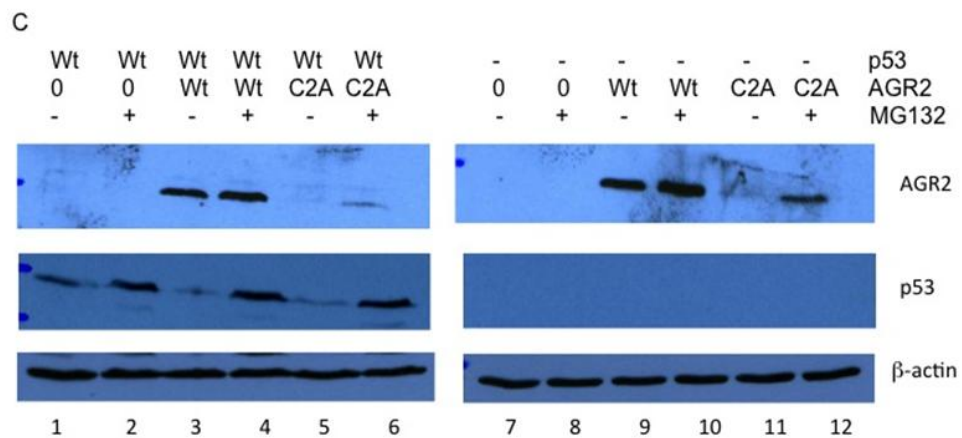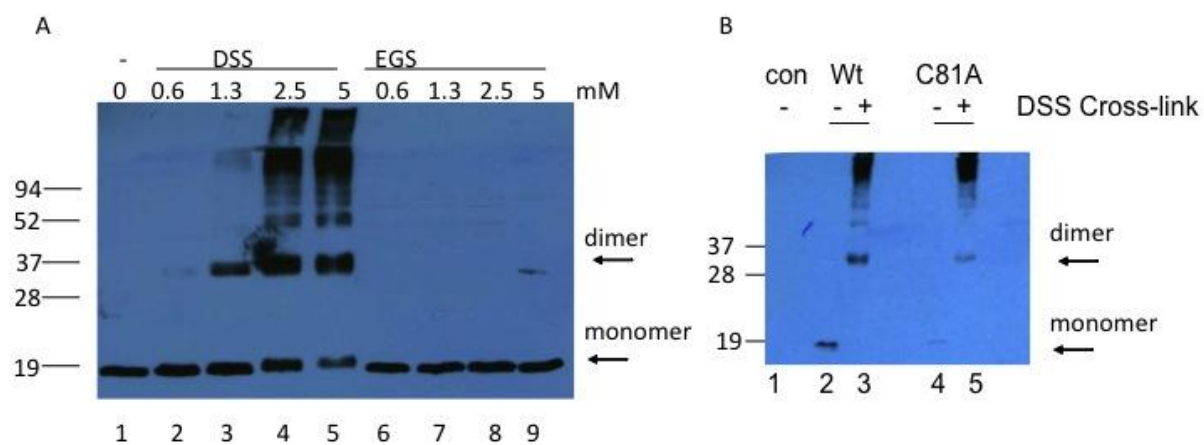
Figure 7. Conversion of AGR2 from monomer and dimer state monitored by mass sepctrometry. (A) Appearance of the 27+ charge state of AGR2 dimer,$[D+ 27H]^{27+}$,as a function of oxidant concentration. Consistent with an AGR2 dimer containing an intermolecular disulfide bond. (B) Isotope distribution of the $[D+ 27H]^{27+}$ charge state of AGR2. The theoretical isotope distribution is overlaid as a scatter plot. (C) Diagram of disulfide bond formation.

Figure 8. Conversion of AGR2 from reduced monomer to monomeric sulfinic/sulfonic acid isoform. (A) Expanded mass-charge analysis of the AGR2 $[D+30H]^{30+}$ /$[M+15H]^{15}$ charge state; Appearance of species consistent with $[M+2O+15H]^{15+}$ (sulfinic acid; highlighted in blue) and $[M+3O+H]^{30+}$(sulfonic acid; highlighted in orange) are observed at higher oxidant concentration. (B) Expanded mass-charge analysis of the $[M+20H]^{20+}$ charge state highlighting the +32 Da mass shift which occurs upon 2.5 mM H_2O_2 treatment of AGR2. (C) This modification is attributed to oxidation of cysteine to sulfinic acid within the AGR2 monomer.

Figure 9. Evidence for sulfenic intermediate in the oxidation of AGR2 protein. (A). Theoretical effects of NBD-Cl modification of cysteine thiols and cysteine sulfenic acids which results in a Δmass of +163 Da. Depicted are the cysteine thiol adduct and the labile sulfenic acid intermediate adduct. (B). Analysis of the $[M+18H]^{18+}$ charge state of of AGR2 protein monomers with distinct cysteine NBD-modifications. AGR2 protein (50 μM) was treated with 4 mM NDB-Cl with out without 0.1 mM H_2O_2. The reaction continued for 45 minutes in the dark prior to quenching with a 3x electrospray make-up solvent containing 45% Methanol and 5% formic acid. The mass/charge spectrum of the $[M +18H]^{18+}$ charge state of AGR2 protein highlights the unmodified protein (top panel); the thiol modified protein (middle panel; 163.963 Da); and a significant amount of the sulfenic-trapped intermediate (bottom panel; 178.982 Da).

18

Figure 10. Distinct isoforms of AGR2 protein can be produced through cysteine oxidation. Oxidation produces a reactive sulfenic acid intermediate that reacts intermolecularly with a reduced AGR2 monomer to form a dimeric covalent complex. Higher levels of oxidant promote the formation of a sulfinic acid that cannot form homodimers or with the highest level of oxidant leading to the formation of the sulfonic acid. These isoforms might have distinct specific activities in protein-protein interactions and in mediating client protein maturation.
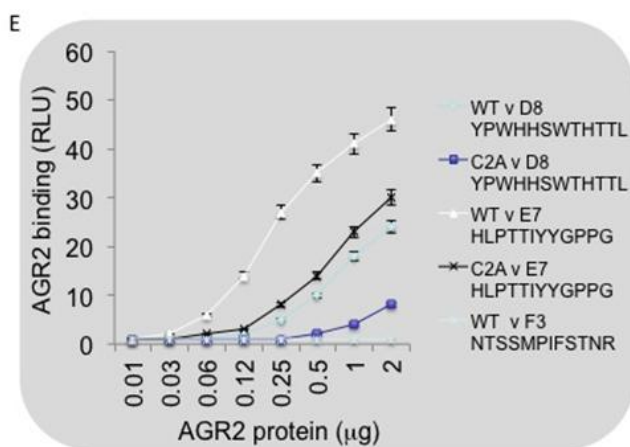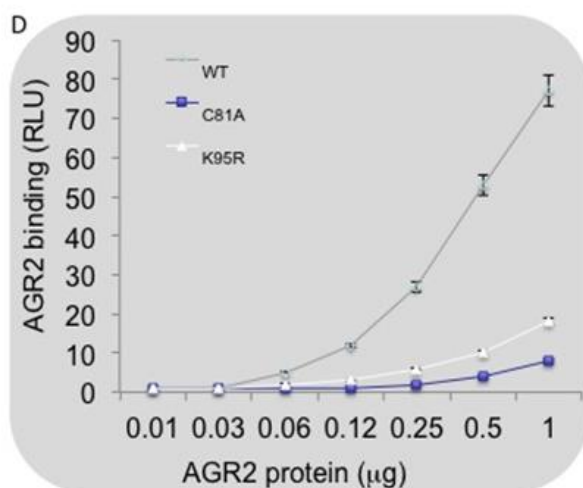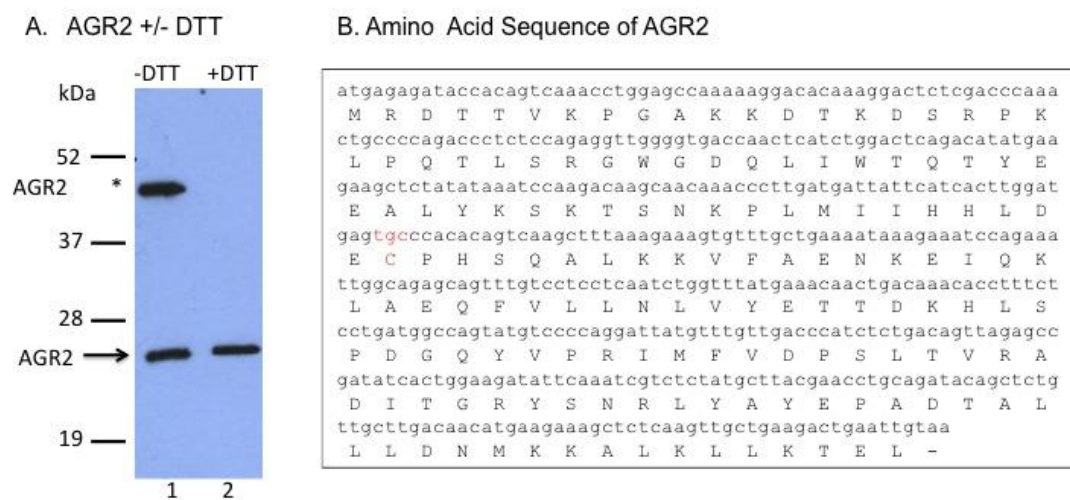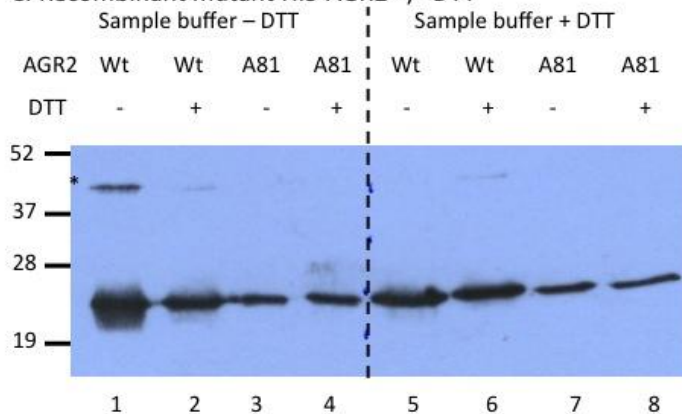
Figure 1

Figure 2

A. AGR2 +/- DTT

B. Amino Acid Sequence of AGR2

```
atgagagataccacagtcaaacctggagccaaaaaggacacaaaggactctcgacccaaa
 M  R  D  T  T  V  K  P  G  A  K  K  D  T  K  D  S  R  P  K
ctgccccagaccctctccagaggttggggtgaccaactcatctggactcagacatatgaa
 L  P  Q  T  L  S  R  G  W  G  D  Q  L  I  W  T  Q  T  Y  E
gaagctctatataaatccaagacaagcaacaaaccccttgatgattattcatcacttggat
 E  A  L  Y  K  S  K  T  S  N  K  P  L  M  I  I  H  H  L  D
gagtgcccacacagtcaagctttaaagaaagtgtttgctgaaaataaagaaatccagaaa
 E  C  P  H  S  Q  A  L  K  K  V  F  A  E  N  K  E  I  Q  K
ttggcagagcagtttgtcctcctcaatctggtttatgaaacaactgacaaacacctttct
 L  A  E  Q  F  V  L  L  N  L  V  Y  E  T  T  D  K  H  L  S
cctgatggccagtatgtccccaggattatgtttgttgacccatctctgacagttagagcc
 P  D  G  Q  Y  V  P  R  I  M  F  V  D  P  S  L  T  V  R  A
gatatcactggaagatattcaaatcgtctctatgcttacgaacctgcagatacagctctg
 D  I  T  G  R  Y  S  N  R  L  Y  A  Y  E  P  A  D  T  A  L
ttgcttgacaacatgaagaaagctctcaagttgctgaagactgaattgtaa
 L  L  D  N  M  K  K  A  L  K  L  L  K  T  E  L  -
```

C. Recombinant mutant HIS-AGR2 +/- DTT

Figure 3

Figure 4

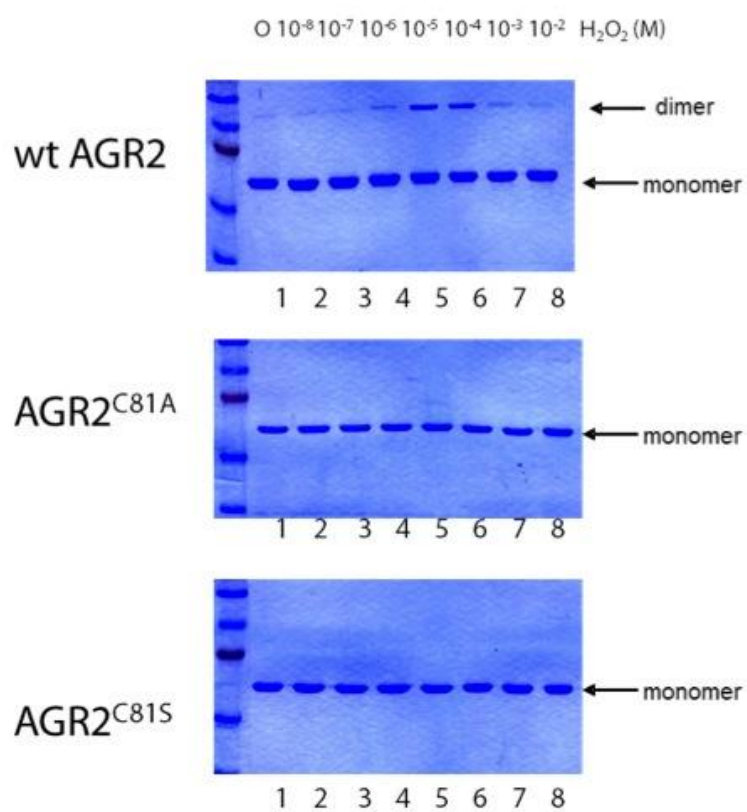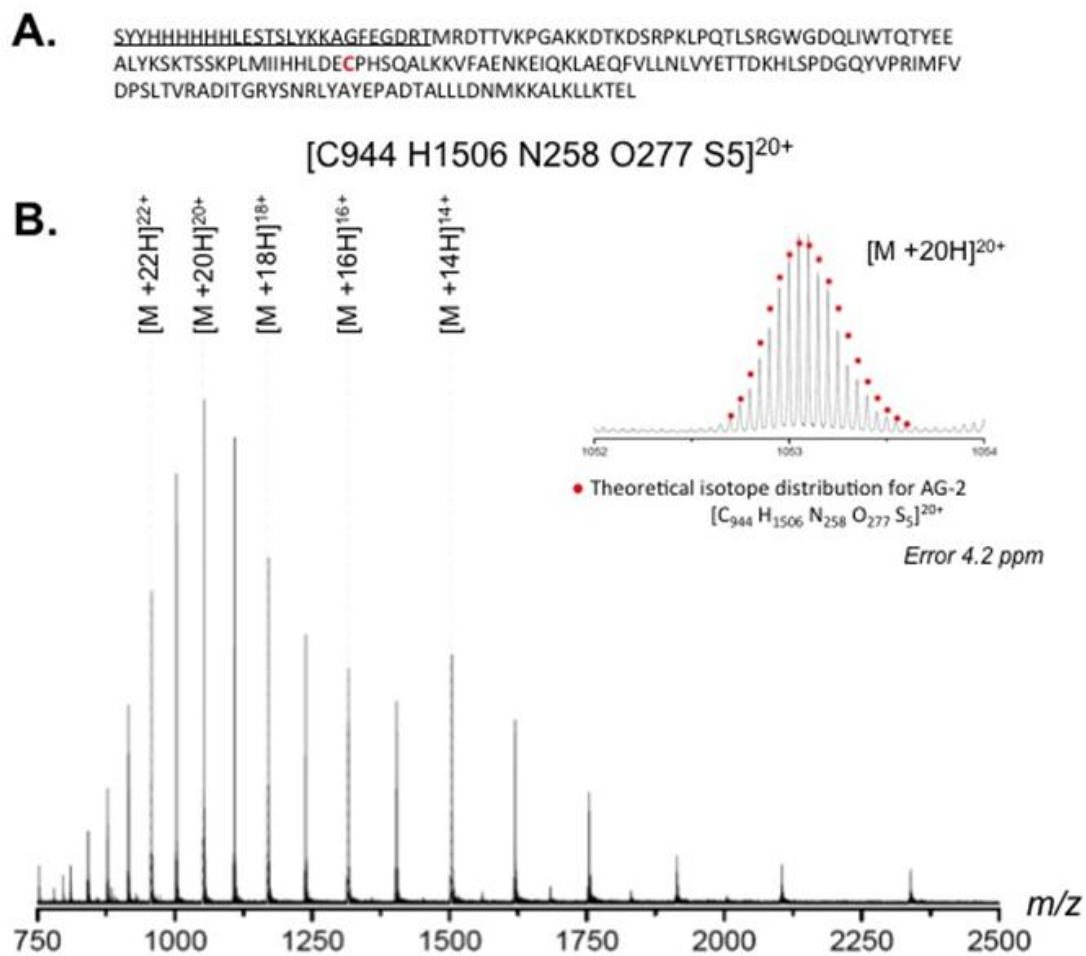$O\ 10^{-8}\ 10^{-7}\ 10^{-6}\ 10^{-5}\ 10^{-4}\ 10^{-3}\ 10^{-2}\quad H_2O_2\ (M)$

wt AGR2 — dimer / monomer
1 2 3 4 5 6 7 8

$AGR2^{C81A}$ — monomer
1 2 3 4 5 6 7 8

$AGR2^{C81S}$ — monomer
1 2 3 4 5 6 7 8

Figure 5

A. SYYHHHHHHLESTSLYKKAGFEGDRTMRDTTVKPGAKKDTKDSRPKLPQTLSRGWGDQLIWTQTYEE
ALYKSKTSSKPLMIIHHLDECPHSQALKKVFAENKEIQKLAEQFVLLNLVYETTDKHLSPDGQYVPRIMFV
DPSLTVRADITGRYSNRLYAYEPADTALLLDNMKKALKLLKTEL

$[C_{944} H_{1506} N_{258} O_{277} S_5]^{20+}$

B.

$[M +22H]^{22+}$
$[M +20H]^{20+}$
$[M +18H]^{18+}$
$[M +16H]^{16+}$
$[M +14H]^{14+}$

$[M +20H]^{20+}$

- Theoretical isotope distribution for AG-2
$[C_{944} H_{1506} N_{258} O_{277} S_5]^{20+}$

Error 4.2 ppm

m/z

750   1000   1250   1500   1750   2000   2250   2500

Figure 6

Figure 7

A.

[D+27H]$^{27+}$

0 µM
6.2 µM
12.5 µM
25 µM
50 µM
100 µM
200 µM
500 µM
2500 µM

1558   1561

B.

1559   1561

● [C1888 H2997 N516 O554 S10]$^{27+}$

C.

AG-2 —SH

↓

AG-2 —S—S— AG-2

Figure 8
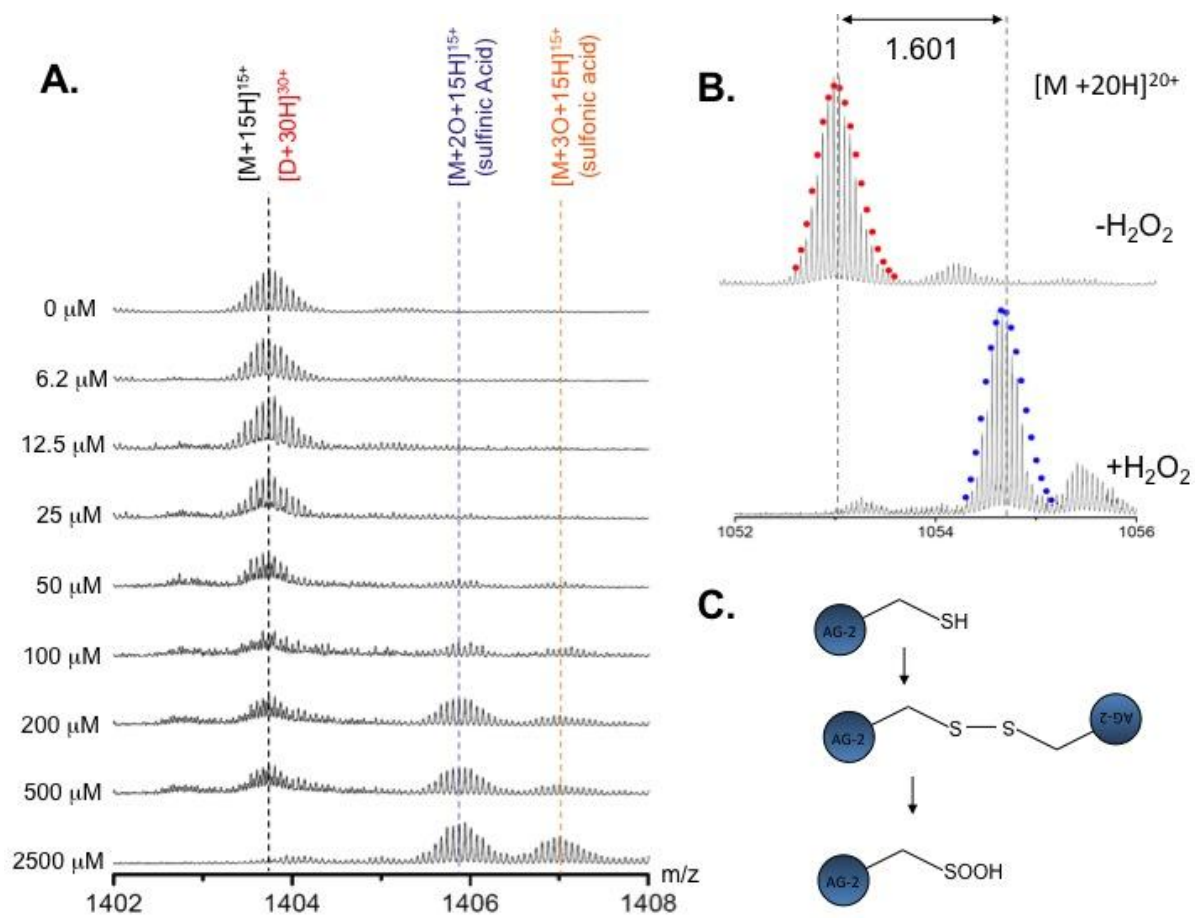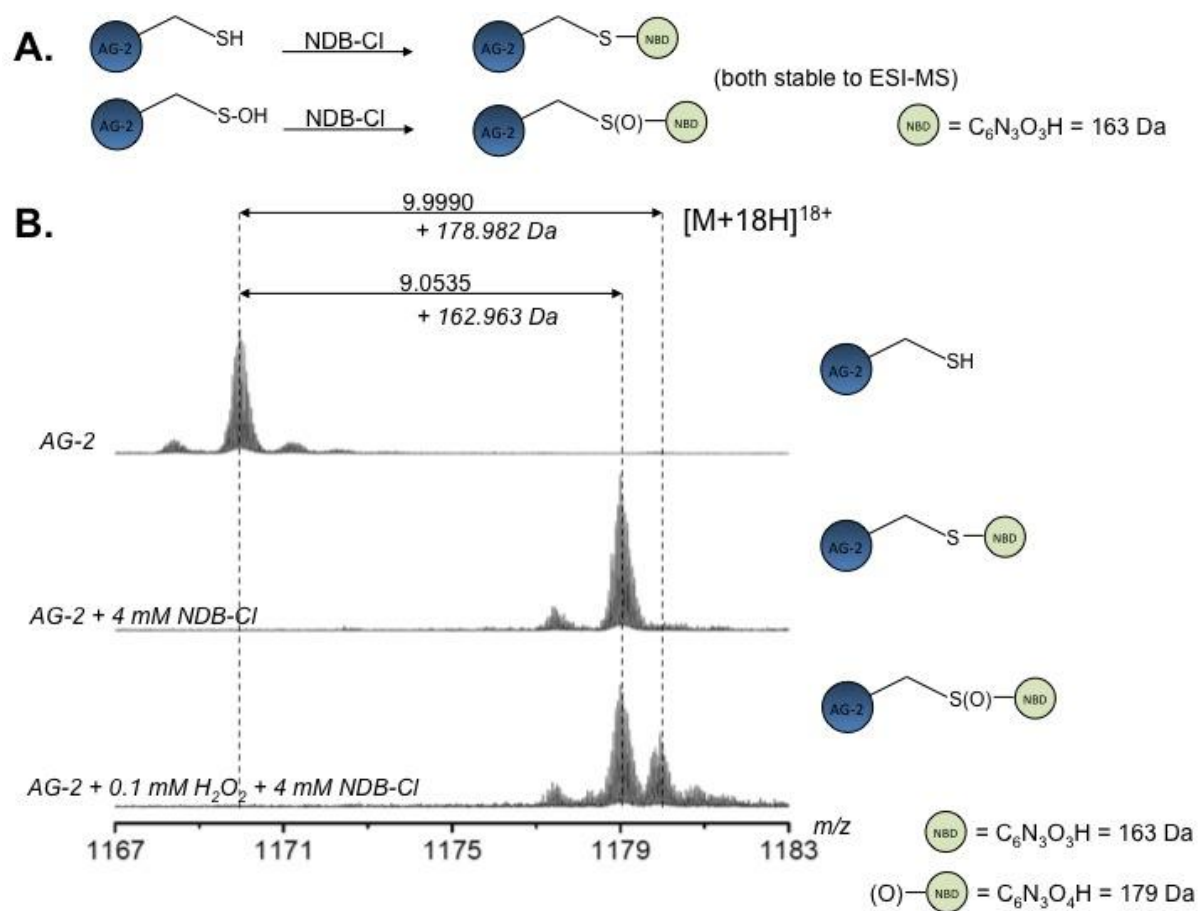
Figure 9
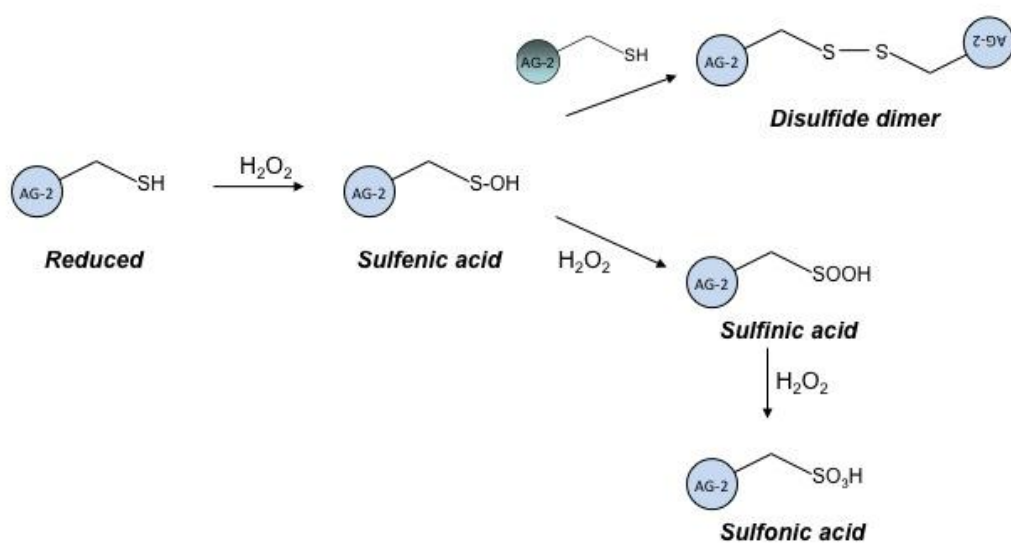
Figure 10

Highlights

- AGR2 is an ER resident chaperone that mediates cysteine-rich receptor maturation
- Mutation of its single cysteine attenuates its protein-protein interaction function
- AGR2 dimerizes by two distinct mechanisms, one of which is through cysteine oxidation
- Oxidation dimerizes AGR2 through a labile sulfenic acid intermediate
- High level oxidation induces a sulfinic or sulfonic acid preventing dimerization
- Differential AGR2 oxidation has the potential to impact on its chaperone function