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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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ABSTRACT

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-disulphide 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 catalysed 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 disulphide 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.

1. 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

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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 [34] like AGR2 have specific roles that enable the folding, trafficking, and assembly of complex cysteine-rich transmembrane 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 affect its specific activity as an oncogenic chaperone in the development and maintenance of the transformed phenotype.

2. Experimental Procedures

2.1. General

All reagents were purchased from Sigma unless otherwise stated.

2.2. 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 37 °C. For cross-linking, cells were grown to ~90% confluence before exposure to DSS (Pierce 21,555) or EGS (Pierce 21,565). DSS or EGS was dissolve in 100% DMSO to a concentration of 100 mM before being further diluted to the appropriate concentration in growth media and added to cells. Cells were incubated for 1 h further at 37 °C before being harvested into PBS and lysed into the following buffer: 150 mM NaCl, 50 mM Tris HCl pH 8.0, 50 mM NaF, 5 mM EDTA, 1% NP-40 plus 1:100 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).

2.3. 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.

2.4. 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 37 °C. One day prior to transfection cells were plated into 6 well plates (2 ml media) so that they would be 90–95% confluent the following day. Plasmid DNA (1 μg) 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 min at RT. After this time the two mixtures were combined, gently mixed and incubated at RT for a further 20 min at RT before addition to cells. Cells were incubated for 24 h before cross-linking was carried out as described above using 1 mM DSS.

2.5. 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 h 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 *Escherichia coli* cells and grown O/N. The cells were subcultured and induced with 1 M IPTG after OD 0.4 had been reached. After 3 h the cells were pelleted and incubated with lysis buffer (10% sucrose, 50 mM Tris (pH 8), 150 mM NaCl, 3 mg Lysozyme, 0.5% NP40, 5 mM DTT, 1 mM Benzamidine, 20 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 1 mM EDTA) for 45 min before a 1-min incubation at 37 °C and sonication on ice. The lysates were then centrifuged at 4000 rpm for 15 min and the supernatant added to glutathione sepharose 4B beads and incubated for 2 h at 4 °C. The beads were washed extensively using wash buffer (10 x with 20 mM HEPES (pH 7.5), 1 mM DTT, 10% glycerol, 150 mM NaCl, ten times with 20 mM HEPES (pH 7.5), 1 mM DTT, 10% glycerol, 1.0 M NaCl, and two times with 20 mM HEPES (pH 7.5), 1 mM DTT, 10% glycerol, 150 mM NaCl) before elution buffer (25 mM HEPES (pH 7.5), 1 mM DTT, 10% glycerol, 150 mM 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).

2.6. 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 min 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.