Seminars in Cancer Biology 33 (2015) 16–24

Contents lists available at ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Review

Mechanisms of anterior gradient-2 regulation and function in cancer

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ARTICLE INFO

Keywords:
AGR2
Endoplasmic reticulum
Endoplasmic reticulum stress
Protein folding
PDI
Cancer
Asthma
Inflammation

ABSTRACT

Proteins targeted to secretory pathway enter the endoplasmic reticulum where they undergo post-translational modification and subsequent quality control executed by exquisite catalysts of protein folding, protein disulphide isomerases (PDIs). These enzymes can often provide strict conformational protein folding solutions to highly cysteine-rich cargo as they facilitate disulphide rearrangement in the endoplasmic reticulum. Under conditions when PDI substrates are not isomerised properly, secreted proteins can accumulate in the endoplasmic reticulum leading to endoplasmic reticulum stress initiation with implications for human disease development. Anterior Gradient-2 (AGR2) is an endoplasmic reticulum-resident PDI superfamily member that has emerged as a dominant effector of basic biological properties in vertebrates including blastoderm formation and limb regeneration. AGR2 perturbation in mammals influences disease processes including cancer progression and drug resistance, asthma, and inflammatory bowel disease. This review will focus on the molecular characteristics, function, and regulation of AGR2, views on its emerging biological functions and misappropriation in disease, and prospects for therapeutic intervention into endoplasmic reticulum-resident protein folding pathways for improving the treatment of human disease.

1. Introduction

1.1. Three paradigms from studies on the biological functions of AGR2

The Anterior Gradient-2 (AGR2) gene is confined to vertebrates [1], suggesting that its appearance in evolution solves specific development problems that opened up in this lineage. Indeed, three distinct types of studies in vertebrates have produced key paradigms on the function of AGR2 and how its misregulation may mediate in human disease. First, *agr2* is a gene whose expression was first noted in *Xenopus laevis* cement glands whereby the protein can sculpt the dorso-anterior ectoderm forming the cement gland and can maintain the forebrain integrity [2,3]. As suggested by the name, the cement gland can mediate the attachment of the developing epithelium to a solid phase [4]; this "adhesion" inducing property of AGR2 foreshadows its potential role in migration and adhesion required for mammalian cancer metastasis [5] [6].

Another key insight into the rather late emergence of AGR2 in metazoans stems from studies in limb regeneration in amphibians. In screening for genes induced in the newt during limb regeneration, the newt homologue of *agr2 (nag)* as identified as a candidate gene and its electroporation into de-nervated limbs was able to restore limb regeneration [7]. Although it is thought that mammals have lost the ability to regenerate appendages, there is a strain of "healer" mouse (MRL) with regenerative characteristics including blastema formation and basement membrane breakdown at a wound site including partial regeneration of amputated digits [8]. Recent studies have shown that mice lacking the cell cycle regulator *p21* gene can mimic some aspects of the MRL, suggesting an inverse correlation between cell cycle progression and AGR2 functions [9]. Indeed, an inverse correlation between AGR2 and p21 expression has been noted previously in mammalian cells [10–12]. This growth promoting property of AGR2 in amphibian limb regeneration forms a paradigm and foreshadows its' future evolutionary role in human cancer cell cycle progression, invasion, and metastasis.

The third key biological description of AGR2 functions came from studies in transgenic mice. Animals that are null for AGR2 are defective in mucin production, have alterations in asthma incidence [13], and susceptible toxin-stimulated inflammatory bowel disease [14]. This provides a compelling paradigm in mammals in which AGR2 can function in epithelial cells as a dominant endoplasmic reticulum localised chaperone that can mediate the folding of cysteine rich client proteins and maintain the secretory landscape

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http://dx.doi.org/10.1016/j.semcancer.2015.04.005
1044-579X/

especially in conditions of stress [15,16]. This property of AGR2 in maintaining the epithelial barrier is further suggestive of how it can function in diseases like cancer; it can be misappropriated in cancers to promote the pro-metastatic secretory landscape important for cancer disseminations and interaction with the local matrix and stroma [17]. Below, we will review the molecular functions in AGR2 and implications in human diseases that fit into these three landmark animal studies on AGR2 signalling.

1.2. Biochemical determinants in AGR2 protein

1.2.1. Three classic linear motifs in AGR2 define its core biochemical determinants

The vast majority of protein-protein interactions in mammals are driven by linear peptide motifs [18]. The primary structure of AGR2 includes such determinants. First, a hydrophobic sequence in its N-terminus (amino acids 1-20) directs AGR2 into endoplasmic reticulum. This sequence is followed by an assumed leader sequence cleavage site between amino acids Ala20 and Lys21, although there is very little information on regulation at the level of proteolysis of its signal sequence by enzymes such as signal peptide peptidase [19]. Signal peptide peptidase can cleave intramembrane signal sequences and, for example, can play a role in ERAD to control the unfolded protein response modulators [20]. Thus, identifying how or whether AGR2 cleavage rates can be affected by signal peptide peptidases and impact upon endoplasmic reticulum quality control, unfolded protein responses, and disease mechanisms remain an interesting future area of research in AGR2 signalling.

The extreme C-terminus includes another classic linear peptide motif; the endoplasmic reticulum retention motif containing tetrapeptide sequence of lysine (K)–threonine (T)–glutamic acid (E)–leucine (L) abbreviated as KTEL [21]. This motif is a non-conventional variant of the classic endoplasmic reticulum localising motif, whose classical form is KDEL [22]. More recent studies have expanded the KDEL motif to include positions 5 and 6 from the C-terminus [23]. The KDEL motif mediates retrograde transport of proteins in endoplasmic reticulum during the protein secretion process into the extracellular space. Proteins possessing KDEL or similar motif interact with KDEL receptors in intermediary compartments of the cis-Golgi complex [24]. The binding results in conformation changes of the KDEL receptor directing the receptor with ligand into vesicles returning to the endoplasmic reticulum. The higher pH in the endoplasmic reticulum results in dissociation of the KDEL peptide motif from the KDEL receptor and its subsequent recycling [23,24].

Mutagenesis of the motif in AGR2 has shown effects in cell based systems; deleting the motif can result in secretion of AGR2 [21], and the KTEL mutation to the canonical KDEL increases the extent of endoplasmic reticulum localisation [12]. In addition, there are functional roles for the KTEL motif in AGR2 with respect to cancer cell survival pathways. The deletion of the motif eliminates its ability to simulate cell growth in clonogenic assays and to attenuate the p53 transcriptional response to DNA damage [10]. These latter data suggest that cancer associated functions of AGR2 stem from its localisation in the endoplasmic reticulum.

Another key linear motif of AGR2 (and its orthologue AGR3) is the CXXS motif or “thioredoxin fold” containing the central and sole cysteine residue through which it can presumably mediate oxidoreductase function [1]. It is this thioredoxin fold that places the gene as a late evolving member of the oxidoreductase family [1]. Other oxidoreductases also harbour CXXS motifs including ERP44 [25,26]. The ancestral founder gene of AGR2/AGR3 exists in invertebrates and is named ERP18 [27,28]. This protein contains the classic di-sulphide thioredoxin fold with the CxxC motif and exhibits classical oxidoreductase activity [28]. ERp18 and AGR2 are not apparently redundant, since it is AGR2 (and not ERP18) that is emerging as oncogenic signalling molecule using OMICS discovery platforms [17]. In addition, AGR2 knock out mice have clear phenotypes indicating its function cannot be replaced by alternate orthologues such as AGR3 or ERp18 [29]. Conversely, AGR3 knockout mice also show a phenotype distinct from AGR2-null animals; in the absence of AGR3, animals are viable but the ciliary beat frequency in lung is lower suggesting a specialised role for AGR3 in mucociliary clearance [30]. Presumably, in the case of AGR2 or AGR3, the single cysteine forms a key non-redundant reaction point in its substrate binding cycle.

Synthetic ERp18 alleles containing a mutation of the CXXC motif to CXXS can covalently trap dithiothreitol-sensitive client proteins [31]. This approach obviously cannot be used to trap di-sulphide sensitive AGR2 intermediates (*e.g.* generating a SXXS mutant); however mutation of the single cysteine in AGR2 can impair mucin interactions in murine models of inflammatory disease [29]. The closest orthologue of AGR2 that resides proximal to the *agr2* gene, *agr3*, also harbours a CXXS motif. The role of AGR3 is more clouded than AGR2, although their expression can be coupled by oestrogen in breast cancers [32] or interestingly uncoupled in ovarian cancers [32]. As mentioned above, studies using AGR3 and AGR2 null mice also point to non-redundant functions in development. Since the single cysteine in AGR2 can obviously impact in its disulphide exchange activity, this residue can form an intriguing target of gene editing to develop a more thorough understanding of how its thioredoxin activity can impact on endoplasmic reticulum homoeostasis and ERAD quality control in normal and diseased states.

In addition to these three linear motifs that drive AGR2 functions, AGR2 can exist in a dimeric structure through a novel amino acid motif (amino acids 60–64; EALYK) that comprises a dimerisation motif [6]. The realisation of this dimeric crystal structure required the deletion of the intrinsically disordered N-terminal 45 amino acids that dramatically increases the affinity of the dimer by many orders of magnitude [6]; *e.g.* the N-terminus plays a natural negative regulatory role to reduce dimer affinity. This suggests a mechanism has evolved to maintain a monomer to dimer equilibrium as physiologically important (Fig. 1). Pharmacological manipulation of AGR2 dimer stability is possible as synthetic peptides from this N-terminal disordered region can regulate the stability of the AGR2 dimer *in trans* [33], suggesting a screening assay to develop drug leads that can alter the AGR2 dimer stability. Indeed, evidence exists that altering dimer stability can affect pro-metastatic properties of the protein [6].

The structure of the dimer opens the door to develop additional insights into AGR2 function. For example, the Cysteine-81 residues in the dimeric structure are held in opposing faces where enhanced disulphide exchange in client proteins might occur [28]. The monomer to dimer equilibrium in AGR2 might therefore be important in disulphide exchange to assemble correctly folded di-sulphide bridges in client cargo proteins; perhaps especially since AGR2 only has one cysteine and the geometry of the dimer might be critical in association and dissociation assays. In addition to this dimeric structure mediated through amino acids 60–64, AGR2 can form an alternatively shaped dimeric structure in which oxidation-dependent homo-dimerisation occurs through Cysteine-81 mediated disulphide bond formation that would re-orientate the dimer into a different conformation [34]. This oxidised homodimer in equilibrium with its canonical dimer interface through amino acids 60–64 might be an important determinant in the chaperonin re-association cycle to control redox state of its client proteins in the endoplasmic reticulum (Figs. 1 and 2).

1.2.2. AGR2 binding proteins

Yeast-two hybrid assays have driven our most comprehensive discovery of AGR2 binding proteins. Such studies have show that