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Review

GRP78: A chaperone with diverse roles beyond the endoplasmic reticulum

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Summary. Glucose-regulated protein 78 (GRP78) is a well-characterized molecular chaperone that is ubiquitously expressed in mammalian cells. GRP78 is best known for binding to hydrophobic patches on nascent polypeptides within the endoplasmic reticulum (ER) and for its role in signaling the unfolded protein response. Structurally, GRP78 is highly conserved across species. The presence of GRP78 or a homologue in nearly every organism from bacteria to man, reflects the central roles it plays in cell survival. While the principal role of GRP78 as a molecular chaperone is a matter of continuing study, independent work demonstrates that like many other proteins with ancient origins, GRP78 plays more roles than originally appreciated. Studies have shown that GRP78 is expressed on the cell surface in many tissue types both *in vitro* and *in vivo*. Cell surface GRP78 is involved in transducing signals from ligands as disparate as activated α_2-macroglobulin and antibodies. Plasmalemmar GRP78 also plays a role in viral entry of Coxsackie B, and Dengue Fever viruses. GRP78 disregulation is also implicated in atherosclerotic, thrombotic, and auto-immune disease. It is challenging to posit a hypothesis as to why an ER molecular chaperone, such as GRP78, plays such a variety of roles in cellular processes. An ancient and highly conserved protein such as GRP78, whose primary function is to bind to misfolded polypeptides, could be uniquely suited to bind a wide variety of ligands and thus, over time, could assume the wide variety of roles it now plays.

Key words: GRP78, Plasma Membrane, Signaling, Unfolded Protein Response, Cell Surface

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Introduction

GRP78 is a 78 kDa molecular chaperone that binds to hydrophobic patches on nascent polypeptides in the ER to prevent aggregation and aid in the development of proper secondary structure of mature proteins. As this function is inescapably necessary in all cells, GRP78 is ubiquitously expressed in mammalian cells. In addition to preventing the physical aggregation of unfolded proteins, GRP78 is one of the initial components in the signaling cascade that results in the unfolded protein response (UPR). As the pool of available GRP78 in the ER is exhausted in binding to unfolded proteins, GRP78 releases secondary signaling mediators that directly act to halt the accumulation of unfolded proteins in the cell or cross the nuclear membrane to initiate transcription of effector proteins for the UPR(Ting and Lee 1988). Studies have since shown that interactions with secondary signaling proteins, including inositol requiring kinase 1 (IRE1), PKR-like ER-associated kinase (PERK), and activating transcription factor 6 (ATF6) regulate the response of GRP78 to Ca^{2+} depletion, glucose and energy depletion, unfolded proteins, toxins, and other events that elicit a response from GRP78 (Shen et al., 2004; Zhang and Kaufman 2006). GRP78 has been identified in many roles outside of the ER in distant cellular sites including the plasma membrane (Xiao et al., 1999; Corrigall et al., 2001; Triantafilou et al., 2001, 2002; Misra et al., 2002; Bodman-Smith, 2003; Bodman-Smith et al., 2003; Arap et al., 2004; Jindadamrongwech et al., 2004; Davidson et al., 2005; Brownlie et al., 2006; Panayi and Corrigall, 2006; Sun et al., 2006; Cabrera-Hernandez et al., 2007). Postulating hypotheses as to how GRP78 can play a role in disparate tasks in these non-ER sites can be challenging as many of these interactions seem to demand very different behavior from a protein classically known as a chaperone. GRP78, however, is an ancient protein that is

capable of binding to a wide variety of nascent polypeptides and transducing a signal with a number of secondary mediators. Such properties may explain how GRP78 plays so many roles. Consideration of the UPR signaling pathway, allows one to propose mechanisms by which GRP78 could serve as a receptor and component of a cell surface signaling pathway. A large body of work now supports these proposed mechanisms.

Study of the role of GRP78 as a master regulator of the unfolded protein response, offers an approach to understanding how it might work as a cell surface receptor. In the ER, GRP78 mediates UPR signaling via three primary signaling partners: IRE1α or IRE1ß, PERK, and ATF6. The known mediators of UPR signaling immediately downstream of GRP78 are all embedded in the membrane of the ER with a luminal ER stress-sensing domain and a cytosolic signaling domain (Cox et al., 1993; Mori et al., 1993; Shi et al., 1998; Harding et al., 1999; Liu et al., 2000; Shen et al., 2002). ER resident GRP78 functions by binding to nascent polypeptides preventing their aggregation before they can be properly folded. GRP78 also directly binds to the luminal portion of IRE, PERK, and ATF6 under normal conditions. During times of stress, the available pool of GRP78 is occupied by preventing the aggregation of a large number of misfolded proteins; the result is that GRP78 releases IRE, PERK, and ATF6 which are then free to transduce the UPR signal (Dorner et al., 1992; Bertolotti et al., 2000; Shen et al., 2002). In this way the GRP78/unfolded protein interaction is not very dissimilar from a receptor/ligand interaction in that through interaction with unfolded proteins GRP78 transduces a signal that results in a response. What makes the interaction of GRP78 with unfolded proteins very different from a receptor ligand interaction is that GRP78 is capable of binding a wide variety of unfolded proteins and theoretically a large quantity of any of them would result in UPR signaling, whereas receptor ligand interactions are more specific and capable of transducing a signal at lower ligand concentrations. GRP78 binding to unfolded proteins does not always result in complete activation of all UPR pathways. Under specific circumstances, such as in B cell differentiation, IRE1α-mediated UPR pathways may be activated but not PERK-mediated pathways (Gass et al., 2002; Zhang et al., 2005). Although the mechanisms for selectivity in UPR signaling are poorly understood the fact that selectivity is possible suggests that conformational changes resulting from GRP78 binding to various unfolded proteins could result in different patterns of signaling.

A number of studies have demonstrated GRP78 expression on the surface of cells from a variety of lineages ranging from cancer cells and endothelial cells to activated macrophages (Xiao et al., 1999; Liu et al., 2003; Davidson et al., 2005; Misra et al., 2005; Misra and Pizzo 2005). Cell surface GRP78 plays a role in activities which include cell signaling, viral entry, and antigen presentation (Triantafilou et al., 2001, 2002; Misra et al., 2002; Jindadamrongwech et al., 2004). Early observations in receptor recognized α_2-macroglobulin (α_2M*) mediated cell surface signaling, demonstrated the existence of a high affinity receptor for α_2M* and signal transduction which was coupled to a pertussis toxin insensitive G-protein (Misra et al., 1994). Later studies demonstrated that the receptor responsible for the increase in inositol triphosphate (IP_3) levels, RAC-alpha serine/threonine-protein kinase (Akt) phosphorylation, NF-κB induction, and the subsequent rise in intracellular Ca^{2+} was GRP78 expressed on the cell surface (Misra et al., 2002; Misra and Pizzo, 2004). This identification posed something of a problem as GRP78 is not known to be a transmembrane protein. In its role in ER signaling, GRP78 is always in the luminal space and IRE, ATF6, and PERK span the ER membrane to facilitate signal transductiod by the observation that cell surface GRP78 is associated with the transmembrane co-chaperone MTJ-1 (Misra et al., 2005) (Xie et al., 2000; Misra and Pizzo, 2008). Ligands bind to GRP78 on the plasma membrane, and several models may explain subsequent signaling behavior. Now well established, is that GRP78 transduces its signal through MTJ-1 signaling complexes (Xie et al., 2000; Misra and Pizzo, 2008). This signal results in an increase in intracellular Ca^{2+} from the extracellular space or the ER. The rise in intracellular Ca^{2+} then results in the UPR signaling observed in response to the addition of α_2M* to 1-LN prostate cancer cells, activated macrophages, and other cells types. However, several studies suggest activation of typical ER-dependent signaling pathways (Misra et al., 2006). While such events may depend on intracellular signaling events, the alternative to this model is the possibility that the full complement of UPR signaling molecules are actually embedded in the plasma membrane as they are in the ER membrane, this is depicted in Figure 1. Since GRP78 is not a trans-membrane protein the signal is then transduced either through IRE1:α ATF 6, PERK, or JTJ-1/Gαq11. The result of either model is that plasma membrane GRP78 signaling resembles the signals sent by GRP78 from the ER with the addition of an increase in IP3, Akt phosphorylation, NF-κB, and a rise in intracellular Ca^{2+}. The significant difference between the two proposed models is whether IRE1, ATF6, and PERK are only found in the ER membrane or are expressed both in the ER membrane and the plasma membrane. A number of approaches have been employed to demonstrate both that GRP78 is on the cell surface and that it is the direct target for ligand-induced signal transduction (Misra et al., 1994a,b, 2002; Asplin et al., 2000). Direct binding studies employing [^{125}I]- α_2M* demonstrate very high affinity binding to a small number of sites (Kd=50-100pM; ~ 1 to 5,000 sites/cell) (Asplin et al., 2000). This binding is blocked by antibodies directed against GRP78. (Misra et al., 2002) In GRP78 knockdown studies, α_2M*-mediated signaling is abrogated (Misra et al., 2006). Moreover, knockdown of MTJ-1, a known binding partner for GRP78 also blocks α_2M* binding

and activation of signaling cascades (Misra et al., 2005). Treatment of 1-LN prostate cancer cells, or other cells as noted above, with α_2M* results in binding of α_2M* to cell surface GRP78, and a resultant signaling cascade that in many ways resembles the UPR. Binding of cell surface GRP78 results in an increase in IRE1α, ATF6, and p-PERK expression as would be expected from activation of the UPR (Misra et al., 2006). Downstream of PERK, eukaryotic translation initiation factor-2α (eIF2α) phosphorylation is seen which would result in transient inhibition of protein synthesis as is seen in the UPR (Misra et al., 2006). Also in line with UPR signaling ATF4 is upregulated resulting in GADD34 upregulation which functions to later restore protein synthesis through inhibition of eIF2α (Misra et al., 2006). Binding of α_2M* to cell surface GRP78 also results in an increase in the UPR signaling molecule ATF6 (Misra et al., 2006). We propose that as in the UPR, activated ATF6(p90) is cleaved by the SP1/SP2 proteases to ATF6(p50), which then upregulates XBP1 mRNA and induces UPR genes in the nucleus. α_2M* signaling through cell surface-associated GRP78 also results in IRE1α upregulation (Misra et al., 2006). We propose that as in UPR signaling IRE1α activation

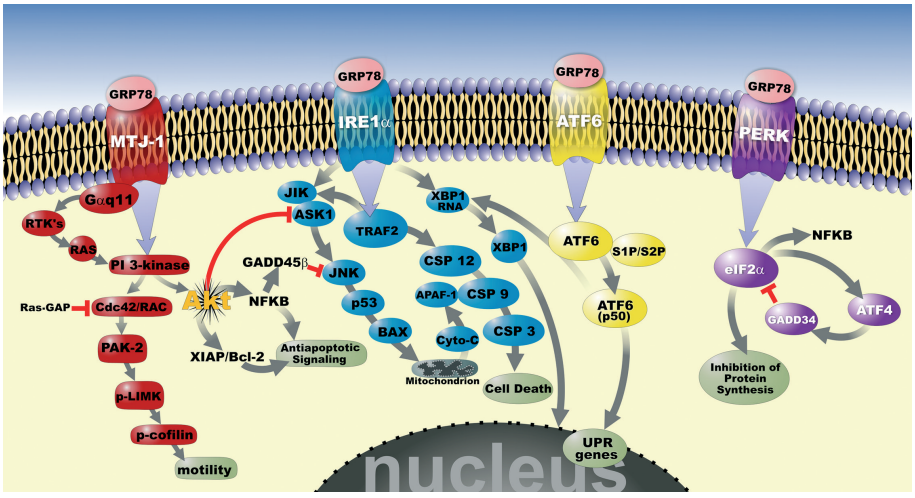

Fig. 1. A model of cell surface-associated GRP78, and its role in signal transduction. Cell surface GRP78-nediated signaling transduced through MTJ-1/Gαq11 is shown in red; this pathway is now well established. Cell surface GRP78-mediated signaling transduced through IREα, ATF6, and PERK is shown in blue, yellow and purple, respectively. The potential role of these pathways in cell surface-mediated signal transduction is currently hypothetical. Biologic Endpoints are shown in light green.

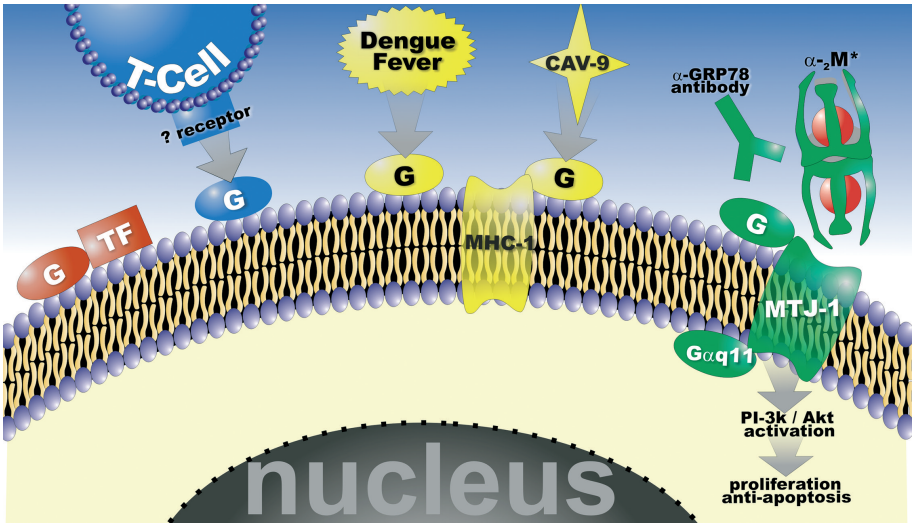

Fig. 2. This figure is an abstraction of all known disease states that relate to cell surface GRP78, regardless of cell type. GRP78 is represented throughout by the oval shape containing only the letter G. GRP78 and its interacting partners are color-coded based on the function of the GRP78 molecule at the surface. The red zone indicates the role of GRP78 in vascular biology. Specifically, GRP78 expression and its association with tissue factor antagonize the pro-coagulant activity of TF without altering its display on the cell membrane. The blue zone depicts the immunosuppressive quality of cell-membrane-bound GRP78. In this model, membrane GRP78 or soluble protein bind to an as yet unknown receptor on T-cells, rendering them anergic. The utilization of GRP 78 as a viral receptor or co-receptor is depicted in the yellow portion of the figure. Dengue Fever Virus has been shown to use GRP78 as a receptor, whereas Coxsackievirus A9 uses GRP78 in association with MCH class 1 molecules. The pathological states that could be perpetrated via ligation of GRP78 as a G-protein coupled receptor are represented in the green zone. Auto-antibodies to GRP78 induce a downstream PI 3-K/Akt α_2M*. The auto-antibodies to GRP78 that arise in some cancer patients should be detrimental due to their pro-proliferative and anti-apoptotic effect on the tumor. In inflammatory conditions and in α_2M*. The resulting ligation of GRP78 and subsequent PI 3-K/Akt signaling could cause proliferation of tumor cells, or in the case of atherosclerosis or RA, proliferation of pathogical tissue types.

results in splicing of XBP1 mRNA. XBP1 mRNA can then be translated to XBP1 which is a transcription factor for many UPR genes. As is seen in UPR signaling, the addition of α_2M* to 1-LN prostate cancer cells results in an increase in ASK1 and TRAF2 (Misra et al., 2006). However, in a departure from what is seen in the UPR, α_2M*- induced upregulation of IRE1α, ASK1, and TRAF2 does not result in an increase in JNK or Caspase 12 activation or the signaling cascades downstream of those proteins leading to cell death (Misra et al., 2006). Through the induction of Akt, which phorsphorylates and inactivates ASK1, and NF-κB, the pro-apoptotic signaling that would be expected from activation of UPR proteins is prevented (Misra et al., 2006). Rather, an upregulation of anti-apoptotic proteins including XIAP, Bcl-2, and 14-3-3 is observed protecting cancer cells from death through the signaling of cell surface-associated GRP78 (Misra et al., 2006; Misra and Pizzo 2008). Another result of signaling through GRP78 and MTJ-1 is the activation of small G proteins, cell division cycle 42(Cdc42), and Ras-related C3 botulinum toxin substrate 1(Rac-1) resulting in phosphorylation of p21 activated protein kinase-2(PAK-2) (Misra et al., 2005). Phosphorylated PAK-2 then causes the phosphorylation of (LIMK) and cofilin (Misra et al., 2005). Rac-1 activation increases cell spreading and migration by stimulating actin polymerization at the plasma membrane and stimulating the formation of lamellipodia. LIMK phosphorylation mediates Rac-induced cytoskeleton reorganization through its associations with the actin cytoskeleton. Phosphorylation of cofilin inhibits its actin depolymerizing activity allowing Rac/LIMK mediated cytoskeletal reorganization to procede. The collective effect of this arm of cell surface GRP78-mediated signaling is a pro-motility phenotype that contributes to the invasive and metastatic behavior exhibited by malignant cancer. This blending of UPR signals with the anti-apoptotic, pro-motility, and pro-proliferative signals mediated by an increase in IP3, Akt phosphorylation, a rise in intracellular Ca^{2+}, and NF-κB induction results in more malignant behavior in cancer cells that express cell surface GRP78.

Cell-surface GRP78 in human disease

The intracellular pool of GRP78 serves an important role in various disease states, and is especially relevant to the rapid and often aberrant protein production associated with neoplasm. Total expression of GRP78 is very frequently greatly increased in dysplastic and malignant tissues with respect to their normal counterparts. An example of this is shown histologically in Figure 3. Increased endoplasmic reticulum-associated GRP78 allows neoplastic cells to withstand the rigors of a tumor microenvironment, as well as conferring a generally more chemotherapeutics-resistant phenotype on different cancer cells. These phenomena have been reviewed extensively elsewhere (Fu and Lee, 2006; Li and Lee, 2006; Panayi and Corrigall, 2006; Lee, 2007; Ni and Lee, 2007).

A growing body of literature indicates the

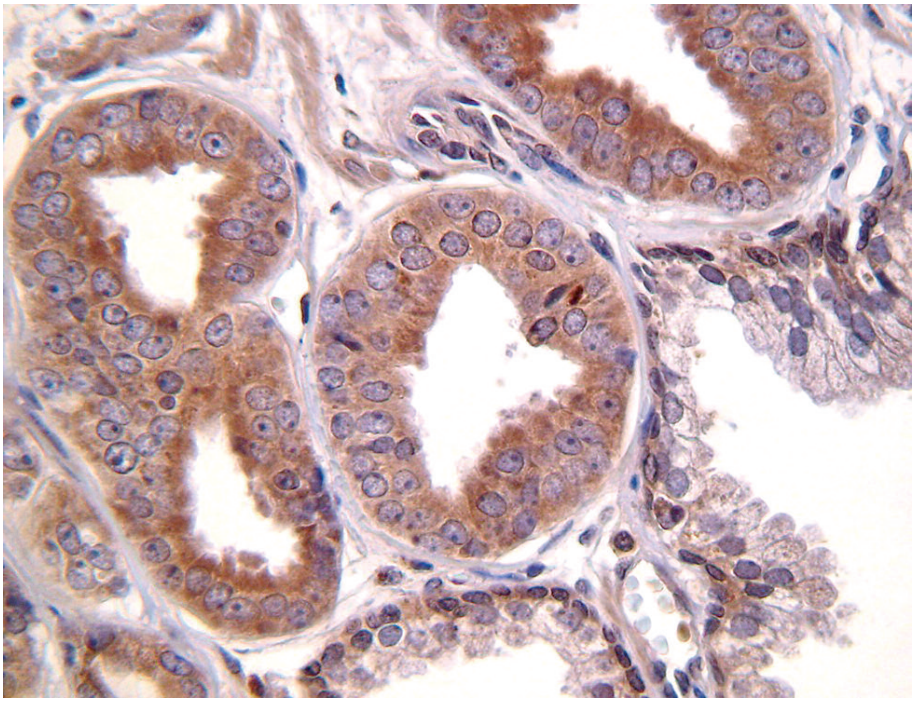

Fig. 3. A typical photomicrograph of a section of a human prostate cancer is shown. Immunohistochemical analysis demonstrates the high expression of GRP78 in cancerous glands. Adjacent non-malignant glands and stroma have notably lower expression of GRP78, some expression is seen in the basal proliferating layers. This photomicrograph was taken with an Olympus BH2 Microscope, with a Canon Powershot GS Digital Camera, at an original magnification of 400x.

phenomenon and significance of GRP78 as a cell-surface receptor and an auto- or neo-antigen in neoplastic disease. In 2003, Arap and colleagues identified GRP78 as a major autoantigen in prostate cancer patients, and presented data that anti-GRP78 antibody occurrence is associated with advanced disease progression and shorter patient survival (Mintz et al., 2003). An *in vivo* study targeting cytotoxic payloads to cancer cells by tagging them with GRP78-binding peptides further validated the significance of surface GRP78 (Arap et al., 2004). This development interestingly mimics a natural phenomenon that results from the binding promiscuity of GRP78. A proteolytic fragment of plasminogen, Kringle 5 (K5), binds to the surface of cancer and endothelial cells and signals apoptosis via a caspase-7 dependent intracellular cascade (Davidson et al., 2005). K5 can interact with cell-surface GRP78, but as demonstrated in the 1-LN prostate cancer cell line, its binding to the voltage dependent anion channel (VDAC) in complex with GRP78 is necessary for signaling to occur (Gonzalez-Gronow et al., 2008). In contrast with the results of K5-VDAC signaling, the antibodies isolated from prostate cancer patients' sera stimulate proliferation and inhibit apoptosis *in vitro* (Gonzalez-Gronow et al., 2006). This *in vitro* effect parallels the relationship of anti-GRP78 titers with negative patient outcomes. How GRP78 is expressed on cancer cell surfaces is likely related to its mechanism of plasma membrane presentation in any cell type. We and others have now demonstrated surface presentation on melanoma, prostate, breast, brain, liver and lung cancer cells (Xiao et al., 1999; Jindadamrongwech et al., 2004; Misra et al., 2005; Gonzalez-Gronow et al., 2006; Kim et al., 2006; Liu et al., 2007). Furthermore, GRP78 is hardly a unique in its deviance from typical subcellular compartmentalization, as it is among three different ER proteins containing the putative ER-retention signal - KDEL localized to the exterior of a brain tumor cell line (Xiao et al., 1999). It is tempting to propose that the formation of anti-GRP78 antibodies in the sera of cancer patients is stimulated by the aberrant cell-surface presentation on many cancer cells of this erstwhile ER-localized protein.

Plasma membrane GRP78 acts as more than a simple bystander in cancer. In a very elegant demonstration of cancer cell marker targeting, Arap and colleagues employed a GRP78-binding peptide fused to an apoptosis-inducing sequence to effect significant killing of breast and prostate cancer cells that express cell surface GRP78. This effect was consistent in murine xenograft and syngeneic tumor models *in vivo* (Arap et al., 2004). Another series of experiments involving a different GRP78-binding peptide with a taxol conjugate yielded similar results in a highly metastatic human melanoma cell line expressing cell-surface GRP78, but not in a low-metastatic reference melanoma line lacking this surface marker (Kim et al., 2006; Liu et al., 2007). In both of these studies, GRP78 ligand internalizion was shown, and this represents a different mode of biological activity than the small g-protein coupled signaling discussed earlier in this review. Very recently, investigation of plasma membrane-associated GRP78 as a binding partner of Cripto, a mitogen associated with various tumors, demonstra (Shani et al., 2008). The intersection of GRP78 membrane biology and TGF-ß in tumor growth and metastasis presents a novel and exciting opportunity for future studies.

Cell-surface GRP78 is also implicated in non-neoplastic human diseases. Through phage-display panning against intact endothelium on *ex vivo* sections of human aortas, Edgington and colleagues demonstrated the presence of GRP78 on endothelial cells overlying atheromatous lesions, but not on neighboring normal vessel wall (Liu et al., 2003). In our original characterization of GRP78 as the α_2M* signaling receptor, we demonstrated a 12.5-fold increase in cell-surface GRP78 (then known as α_2M* signaling receptor) on thioglycollate-elicited murine peritoneal macrophages, as compared to resident peritoneal macrophages (Bhattacharjee et al., 2001). More recently, we have determined that primary human aortic smooth muscle cells express functional surface GRP78, as determined by Akt phosphorylation subsequent to α_2M* ligation, and that surface GRP78 expression is increased by exposure to homocysteine (unpublished results). In light of the mitogenic role of GRP78 signaling and the proliferative nature of atheroma development, it is intriguing that endothelium, macrophages, and smooth muscle, all major components of atheromas, all express surface GRP78 in a stressed state, such as might exist within an atheroma. The relevance of cell surface GRP78 to vascular disease goes beyond atheroma wall formation, however.

Tissue factor (TF) acts as the major cell-surface effector of the extrinsic pathway of coagulation via binding to the serine protease factor VII/VIIa and leading to factor IXa and Xa generation and thus eventually to thrombin activation. Dysregulation of this protein has been implicated in a variety of diverse vascular pathologies such as atherosclerosis, venous thromboembolism, antiphospholipid syndrome, and sepsis-triggered disseminated intravascular coagulation (Taubman, 1993; Logan et al., 1994; Amengual et al., 1998; Asada et al., 1998). TF-related biology and pathology has been studied and reviewed extensively. The interaction of GRP78 and TF activity represents a potentially significant field for further study.

The overexpression of GRP78 in a bladder cancer cell line and cultured human aortic smooth muscle cells negatively influences TF pro-coagulant activity in response to a number of TF stimuli, including $Ca2_+$ influx, hydrogen peroxide, and adenoviral infection (Watson et al., 2003). A counterintuitive but intriguing finding in this study was that GRP78 overexpression did not alter the expression or cell surface display of TF while diminishing its pro-coagulant activity. Further investigation by Bhattacharjee and colleagues revealed through anti-GRP78 antibody treatment of intact murine

brain endothelial cells (bEND.3) and macrophage-like RAW cells that GRP78 inhibition at the cell surface significantly increased TF activity (Bhattacharjee et al., 2005). This result suggests a physical interaction of GRP78 and TF at the cell surface, and indeed, these authors were able to demonstrate this via reciprocal co-immunoprecipitation from whole cell extracts and biotinylated cell membrane preparations of bEND.3 and RAW cells. In summary, it seems that the interaction of GRP78 and TF in the vascular milieu yields important advancements in understanding the basic biology of multiple disorders. The GRP78-TF regulatory axis may also provide novel targets for pharmacotherapy in atherosclerosis, antiphospholipid syndrome, and disseminated intravascular coagulation.

GRP78 plays a role in certain primary autoimmune disorders, including the most frequently debilitating one, rheumatoid arthritis. GRP78 was identified as an autoantigen in RA patients by Corrigall and colleagues in 2001, associated with both antibody and T-cell proliferation in response to soluble GRP78, and furthermore demonstrated GRP78 tolerogenicity in collagen-induced arthritis (CIA) in mice and adjuvant arthritis in rats. GRP78 treatment was thus shown to prevent induction of experimental arthritis (Corrigall et al., 2001). Priming and reactivity to GRP78 in RA patients and normal controls seems to correlate with a TH2-type cytokine profile, and IL-10 producing T-cells responsive to GRP78 were selected and cloned from normal individuals (Bodman-Smith et al., 2003). This group furthered this research by actually treating mice in the active phase of CIA with subcutaneous or IV GRP78 injection, and this effect was recapitulated by passive transfer of GRP78-specific, IL-4-secreting regulatory T-cells. The therapeutic effect of GRP78 administration was abrogated in IL-4 knockout mice (Brownlie et al., 2006). From our own work, a prospective study of 15 patients with RA demonstrated that treatment with anti-TNF-alpha antibody (adalimumab) decreased anti-GRP78 serum antibody titers by over 50% while significantly reducing RA disease activity in terms of DAS28 scores (Mavropoulos et al., 2005). Anti-GRP78 antibodies have also been shown to correlate with primary Sjögrens syndrome (Bodman-Smith, 2003) as well as polyarticular, rheumatoid factor positive Juvenile Idiopathic Arthritis in children (Bodman-Smith et al., 2004).

Finally, the possible role of GRP78 in viral infectious diseases deser (Triantafilou et al., 2001, 2002). These studies indicated physical interaction between CoxA9 particles and cell-surface GRP78, but not between virus and MHC1. On the other hand, by reconstituting Daudi cells with ce receptor for Dengue virus serotype 2 (Jindadamrongwech et al., 2004). In primary monocytes, monocytic cell lines, and neuroblastoma cells, a HSP70/HSP90 complex has been described as an alternate Dengue receptor system (Reyes-Del Valle et al., 2005), however, the specificity of GRP78 for this function, independent of HSP70 and HSP90 has been confirmed (Cabrera-Hernandez et al., 2007).

Clearly, GRP78 is a frequent player in neoplastic, inflammatory and autoimmune disease and viral infection, and its role is manifold: we have described a role as a mitogenic surface adapter for small G-protein signaling via the PI3K-Akt axis, GRP78 modulates the UPR by binding the UPR transcription factors at the ER membrane, and we propose a model whereby this UPR modulating function is recapitulated at the cell membrane in much the same manner as happens in the ER. Immunologically, there is good evidence that GRP78 becomes an autoantigen in cancer, atherothrombotic diseases, and autoimmune inflammatory disorders such as RA. Finally, the function of GRP78 as receptor for the unrelated Dengue and Coxsackie viruses in addition to so many other proteins leads to the suggestion that first, GRP78, a truly ancient protein, serves a surface function as a sensor of degraded, misfolded, or foreign proteins.

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