Identifying Insulin Granule Proteins

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Only four studies have attempted to investigate ISG proteins by proteomic analysis to date. These studies employ various combinations of density gradient centrifugations, in silico analyses, and immunoprecipitation techniques . As a result, Li and colleagues identified 81 total ISG proteins from the INS-1 rat beta-cell line, while Schvartz et al. identified 140 ISG proteins, Hickey et al. identified 51 ISG proteins, and Brunner et al. identified 130 ISG proteins from the INS-1E rat beta-cell line. Proteomic data obtained from these four studies on ISG proteins from INS-1 or INS1-E cells produced a total of 5 proteins that were consistently identified. These were: Insulin-1 (Ins1), Insulin-2 (Ins2), Carboxypeptidase E (CPE), Chromogranin-A (CgA) and Prohormone convertase 2 (PC2). Rat beta-cells synthesize two different forms of insulin encoded by the Ins1 and Ins2 gene that share 90% homology [66,67], hence two insulin forms found in these proteomes. Though different isolation techniques would influence the proteins identified, one would expect that using similar cell lines would result in more than a handful of proteins consistently identified across all four studies.

Keywords: insulin secretory granule ; beta-cells ; granule protein purification

1. Intravesicular Proteins

The most consistently identified intravesicular proteins in the proteomic studies were the previously well-characterised ISG proteins insulin (Ins1 and Ins2), CPE, PC2 and CgA [1][2][3][4][5]. Discovery of proinsulin processing of labelled insulin [6] and CgA [7] have allowed subsequent studies to identify localization of PC1/3 [8], PC2 [9] and CPE [10] as ISG localized enzymes. While all proteomes identified PC2 and CPE, PC1/3 was discovered only in two studies [11][12]. Other intravesicular proteins identified were from the chromogranin-secretogranin protein family. CgA in particular was identified in all four studies, with full-length CgA believed to be important for the biogenesis of granules in beta-cells [13]. Interestingly, CgA knockout mice display a reduced islet number, beta-cell to alpha-cell ratio and plasma insulin levels [14]; however, they exhibit normal blood glucose levels, as a result of compensation from other granin proteins [3]. CgB has been suggested to not be specifically involved in granule formation but instead is essential in the secretion of insulin and other islet hormones such as somatostatin and glucagon [15]. However, through pulse-chase labelling of CgB, Bearrows et al. show that in the absence of CgB, there is a delay in proinsulin trafficking from the TGN followed by a reduction in nascent ISGs at the plasma membrane [16]. CgB was identified in three of the four ISG proteomes (all but Li et al.). Significantly, aside from the full-length granins, PC1/3 and PC2 also cleave granins to form active peptides [4][17]. Beta-granin is an example of a CgA derived peptide identified by Li et al. and is proposed to inhibit insulin secretion through unknown mechanisms [18]. This emphasises technical challenges in peptide identification in proteomics analysis, to differentiate the presence and eventual function of both granins and their derived peptides in future studies.

Hydrolases were found in two of the proteomics analyses [19][20]. Cathepsins B and L were identified by Brunner et al. and are most intriguing as these proteins have been previously shown by electron microscopy to localise in immature ISGs, while cathepsin L alone remains in mature ISGs [21]. While some hydrolases have previously been described within ISGs [22][23], other hydrolases present in proteomic analysis may be appearing due to crinophagy processes of ISGs with lysosomes [20][24]. As such, further validation of hydrolase proteins will be essential to help elucidate their role in ISG biogenesis and processing. Particularly, the validation of cathepsins present in immature and mature ISGs demonstrates that these enzymes may follow sorting mechanisms out of immature ISGs via the mannose 6-phosphate receptor [21][25]. This adds weight to the '*sorting by retention*' and '*sorting by exit*' hypotheses in ISGs, in which immature ISGs may target proteins either for retention in maturing granules or exit towards the lysosome [21][20][26].

2. Membrane Proteins

A substantial proportion of ISG proteins identified by the proteomic analyses were membrane-bound or membrane-associated proteins. Of this group, the most commonly identified were synaptobrevin proteins (VAMPs), including Vamp3 [11][20][12], Vamp7 and Vamp8 [20]. VAMPs interact with their cognate t-SNAREs and other proteins that mediate the fusion

of vesicles to the target membrane [27][28], which in turn interact with a variety of presynaptic proteins and q-SNAREs to form the complete SNARE complex [29][30][31]. Vamp2 was first described as an ISG localised v-SNARE protein [32] by cDNA cloning and confocal microscopy. Brunner et al. then identified Vamp2 in their proteomics analysis and following this, Hickey et al. used Vamp2 antibodies to immuno-purify ISGs. Surprisingly, Hickey et al. and Li et al. do not identify Vamp2 in their proteomes, with Hickey et al. suggesting that it and many other docking proteins potentially remained on the immunoaffinity beads [19]. If these membranal proteins were left unidentified, this may explain why fewer proteins (51) were identified in comparison to other proteomes.

Rab proteins were also found to be enriched with ISG fractions. Rab proteins are a family of GTPases from the Ras superfamily [33] that modulate several stages of vesicle trafficking and fusion of ISGs with the plasma membrane [34][35]. Through proteomic analysis and colocalisation imaging, Brunner's study illustrated that both VAMP8 and Rab37 are novel ISG associated proteins that colocalise with ISGs of INS1-E cells [20]. Previous to this, only 30 proteins were described as ISG associated proteins in beta-cells [20] and information surrounding the trafficking of ISGs was limited. Their proteomic analyses and validation of novel proteins suggested a more complex trafficking process than previously established in beta-cells. Other SNARE complex proteins present in the proteomes include syntaxin5 and 12, (Stx5, Stx12) [11] and granuphilin [20]. However, these proteins are believed to be localised to the plasma membrane [36] and not on ISG membranes, suggesting that they were present in contaminant co-purification with ISG fractions.

Many ATPase subunits were commonly identified in the four proteomic analyses, most notably the vacuolar-H^+ ATPases (V-type). These V-type ATPases have been previously shown to be localized to ISGs in beta-cells [37], and are important in producing and maintaining a proton gradient by acidifying the granule [37][38][39]. This facilitates the maturation of ISGs [40] as well as maintaining a suitable pH for intravesicular enzymes [41][24]. Many other subunits of ATPases identified are lysosomal isoforms and should be validated as to whether they are genuine ISG proteins or proteins co-purified with ISGs.

3. Other Proteins

The remaining proteins identified with non-specific or unknown localization in ISGs are often grouped in these studies. These include cytoskeletal, cytoplasmic and organelle localized proteins. The cytoplasmic proteins identified range from mis-folding chaperones [19] and isomerases (PDIA3) [20] to N-ethylmaleimide sensitive fusion protein [11][12]. Whether these proteins are genuinely ISG-associated, or technical contaminants, requires further validation. Different cytoskeleton-associated proteins are found across all four proteomes. Alpha-centractin [12], alpha and beta-actin [19] and kinesin subunits [12] are some examples of cytoskeletal associated proteins identified. ISGs are transported along microtubules by kinesins [42] and cytoskeleton remodelling is critical for ISG trafficking during glucose-stimulated insulin secretion [43]. The presence of these proteins is therefore unsurprising, though are likely present due to co-purification of these proteins through the isolation of ISGs. Indeed, the presence of proteins localized to the ER, Golgi, mitochondria and lysosomes are also commonly observed across all four studies. Examples include Erp44 (ER), Glg1 (Golgi), SHMT (mitochondria) and Lamp1 (lysosomes) [11][12]. It is difficult to prevent the copurification of these proteins using present isolation techniques and their co-localisations with ISGs need further validation.

The presence of isomerases and proteins involved in protein folding is quite surprising. Hickey et al. in particular find a striking number of chaperone proteins (~20% of proteins identified) [19]. Recent studies have shown that ER chaperone proteins are vital in proinsulin handling and insulin-like growth factor folding [44]; however, none of these ER-resident proteins have been shown to be localized in ISGs. Interestingly, Stanniocalcin-1 (STC1) or its precursors were found in three of the four proteomes (Li, Schvartz, Brunner). STC1 is found in many tissue types such as muscle, kidney, adrenal and lung [45]. Human STC1 protein is described as an uncoupler of oxidative phosphorylation in mitochondria [46], and has been implicated in apoptotic mechanisms and carcinogenesis [47]. Its function in beta-cells is not well understood, however; immunocytochemistry, and *in situ* ligand binding and hybridization [48] show that STC1 colocalizes with insulin in mouse pancreatic beta-cells. The abundance of these chaperones, alongside identification of proteins such as STC1, illustrates the importance of ISG proteomics as a rich source of data to potentially identify novel ISG proteins that may modulate different processes of ISG biogenesis, trafficking, and secretion. Altogether, these studies highlight the importance of developing improved purification techniques that restrict isolation of ISGs to granules post-sorting and packaging from the TGN, and before degradation.

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