Identification of PDI family members required for the production of useful proteins

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Research aims

A disulfide bond is a covalent linkage formed by two cysteines on a protein. Formation of disulfide bonds is catalyzed *in vivo* and is a critical step in the folding of a great number of secretory and membrane proteins[1)]. Many of commercially valuable mammalian proteins such as human growth hormones are secretory proteins with disulfide bonds. A major obstacle for the production of recombinant secretory proteins of mammalian origin in microorganisms is the difficulty in forming correct disulfide bonds. This problem is probably caused by differences in the protein disulfide bond formation systems of microorganisms and mammalian cells. In the endoplasmic reticulum (ER) of mammalian cells, for instance, there are ~20 protein disulfide isomerase (PDI) family members that are thought to catalyze the formation of disulfide bonds in secretory and membrane proteins. The high number of different enzymes probably reflects the need to introduce disulfide bonds into a great variety of secretory proteins present in mammalian cells. However, the physiological roles of many of the PDI family members remain unclear. Insulin, a peptide hormone produced by pancreatic β-cells, plays an important role in the maintenance of blood glucose levels. Insulin has three disulfide bonds. For the folding and activity of insulin, it is essential that all three disulfide bonds are correctly formed. However, the enzyme(s) responsible for this process remain unclear. To identify PDI family members that are required for insulin production, we explored the physiological substrates of PDIp, a PDI family member that is specifically expressed in the pancreas, and identified the PDI family members that directly associate with proinsulin.

Methods

During catalysis of disulfide bond formation, PDI family members form a disulfide-linked complex with their substrate[1)]. We have established methods to stabilize such enzyme-substrate complexes that arise in cultured cells or in animal tissue by treating samples with 10% trichloroacetic acid and 50 mM *N*-ethylmaleimide[2)]. After the enzyme-substrate complexes were purified using an antibody, they were subjected to mass spectroscopic analysis for the identification of either the substrates of PDIp or PDI family

member(s) that directly interact(s) with proinsulin.

Results

Identification of the substrates of PDIp, a PDI family member specifically expressed in the pancreas

PDIp is a PDI family member specifically expressed in the pancreas, which produces hormones such as insulin and digestive enzymes such as trypsin. However, the physiological functions of PDIp remained unclear. Here, we aimed to identify the endogenous substrates of this enzyme in order to understand its function. In particular, we wished to know whether or not PDIp plays a role in the production of insulin. By combining acid quenching and thiol alkylation, we stabilized and purified the complexes formed between PDIp and its substrate proteins from mouse pancreas. Further mass spectrometry analysis of the complexes enabled us to identify the disulfide-linked partners of PDIp *in vivo* and find that PDIp interacts directly with pancreatic digestive enzymes but not with pancreatic hormones such as insulin and glucagon. Pancreas consists mainly of endocrine cells that produce hormones such as insulin and exocrine cells that produce digestive enzymes. We separated mouse pancreatic endocrine and exocrine cells and studied the distribution of PDIp using an anti-PDIp antibody to find that PDIp is expressed in the exocrine cells but not in the endocrine cells, thereby excluding the possibility that PDIp may play a role in the production of insulin. Interestingly, when one of the identified proteins was expressed in cultured cells, its proenzyme formed disulfide-linked aggregates within cells. However, co-expression of PDIp with the protein prevented the formation of aggregates and enhanced the secretion of proenzyme into the medium. Strikingly, we failed to see such effect when we co-expressed other PDI family members with the protein. Thus, PDIp is likely a PDI family member that has evolved to assist in the biosynthesis of digestive enzymes[3)]. These findings should provide important information for the analysis of the causes of diseases related to the pancreas and for the establishment of an efficient system for the production of digestive enzymes of human origin in microorganisms.

Identification of PDI family members that directly interact with proinsulin

Disulfide bond formation in insulin takes place while insulin exists as proinsulin in the ER of pancreatic β-cells. Upon folding, proinsulin is transported to secretory vesicles and processed to form mature insulin. As we have already described above, during catalysis of disulfide bond formation, PDI family members form disulfide-linked complexes with their substrates. We adopted this property to identify the enzymes involved in insulin disulfide bond formation. By treating an insulin-producing pancreatic β-cell line, MIN6, with trichloroacetic acid and *N*-ethylmaleimide, we stabilized the disulfide-linked complexes

between (a) PDI family member(s) and proinsulin[4]. We then purified the complexes using an antibody against proinsulin and analyzed them by mass spectrometry. This analysis led to the discovery of four PDI family members that directly interacted with proinsulin via (an) intermolecular disulfide bond(s). Interestingly, the phenotype obtained by knocking down one of these proteins in MIN6 was different from that obtained by knocking down another protein, suggesting that the two enzymes acted at different steps in the folding of proinsulin.

Conclusion

In this study, we discovered that PDIp is required for the biosynthesis of digestive enzyme(s), but not for that of insulin. Although PDIp is evolutionarily conserved among vertebrates, it remained unclear why this enzyme is expressed specifically in the pancreas. We have elucidated this with our current study[3]. Furthermore, these findings should aid the establishment of an efficient system for the production of digestive enzymes of human origin in microorganisms.

We have also successfully identified four PDI family members that interact with proinsulin via an intermolecular disulfide bond(s) using a pancreatic β–cell line. Interestingly, the phenotype obtained by knocking down one of these proteins in the cell was different from that obtained by knocking down another protein, suggesting that the two enzymes acted at different steps in the biosynthesis of insulin. The findings obtained in this study provide an important basis for future studies.

References

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