Expression of the adrenomedullin binding protein, complement factor H, in the pancreas and its physiological impact on insulin secretion

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Abstract

Adrenomedullin (AM) is a ubiquitous peptide hormone which, among other functional roles, reduces insulin secretion in the pancreas. Recently we have described the interaction between AM and the complement regulator protein factor H, which results in mutual modulation of their respective functions. Here we identify the expression of factor H in the β cells of the rat pancreatic islets by immunohistochemistry and multiple immunofluorescence followed by confocal microscopy. In addition, double immunogold staining under the electron microscope showed coexistence of insulin and factor H immunoreactivities within the same secretory granules; interestingly, factor H staining was found in the electron-lucent

haloes whereas the insulin antibody labeled preferentially the dense cores. The existence of factor H mRNA in the pancreas was confirmed by RT-PCR and *in situ* hybridization. The function of factor H in the pancreas was investigated with an insulin secretion assay. Addition of factor H to freshly isolated islets in the presence of AM resulted in a further reduction in insulin secretion with a concomitant elevation of cAMP, suggesting that factor H increases AM function in glucose homeostasis. The expression of factor H in the pancreas may play other important roles such as protection against complement-mediated cell lysis.

Journal of Endocrinology (2001) **170**, 503–511

Introduction

Adrenomedullin (AM) is a recently recognized pancreatic hormone which is produced in the periphery of the islets of Langerhans, in the same cells that synthesize and secrete pancreatic polypeptide in mammals (Martínez *et al.* 1996). AM, as well as the other members of its peptide family, amylin and calcitonin gene-related peptide (CGRP), exerts an inhibitory role on glucose-stimulated insulin secretion (Martínez *et al.* 1996) and, in some cases, may even be responsible for triggering type 2 diabetes (Martínez *et al.* 1999). This tonic inhibition of insulin release is mediated through a variety of seven-transmembrane domain G-protein coupled membrane receptors that have been found in the insulin-producing β cells (Martínez *et al.* 2000). Pancreatic AM seems to have an early origin from both the phylogenetic and ontogenetic points of view. AM has been found in the pancreas of all vertebrate classes, exhibiting a complex pattern of colocalization with other pancreatic hormones, although in some cases it seems to identify a new cell type which contains AM only (López *et al.* 1999). During embryonal development AM is also present from the very beginning

of pancreatic differentiation, indicating some crucial function for this peptide hormone in embryogenesis (Martínez *et al.* 1998).

Recently, we have identified an AM binding protein in the plasma of several species with an apparent M_r of 120 kDa under non-reducing conditions (Elsasser *et al.* 1999). When this binding protein was purified to homogeneity and sequenced, database analysis identified our isolated protein as complement factor H, an inhibitor of the alternative pathway of the complement cascade (Pío *et al.* 2001). Interestingly, factor H had different effects on AM physiology depending on the assays performed. AM-induced increase of cAMP in Rat2 fibroblast cells was augmented in a dose-dependent manner by the exogenous addition of factor H, even though factor H by itself did not elicit any cAMP response. Factor H was also shown to elevate the growth stimulatory effect of AM in a breast cancer cell line. On the other hand, addition of factor H to a receptor-independent AM bactericidal assay resulted in lower killing efficiencies. Interestingly, AM was demonstrated to modify factor H function by increasing its cofactor activity in the cleavage of C3b by factor I (Pío *et al.* 2001).

Given the importance of AM in the regulation of insulin secretion, we decided to study the presence of its binding protein in the pancreas and determine the physiological impact of factor H in insulin release.

Materials and Methods

Immunohistochemistry

Six male albino Wistar rats were euthanized with CO₂, and perfused with the fixative (4% paraformaldehyde) through a heart cannula. The pancreata were extracted and immersed in the same fixative for an additional period of 5 h. After dehydration, the tissues were embedded in paraffin and sectioned following routine procedures.

Two commercially available polyclonal antibodies against human factor H were used in conjunction with an avidin–biotinylated peroxidase detection kit (Dakopatts, Glostrup, Denmark). The antibodies were goat anti-human factor H (Quidel, San Diego, CA, USA) and rabbit anti-human factor H (Serotec, Raleigh, NC, USA). To ensure the specificity of the signal, liquid-phase and solid-phase absorption controls were performed with purified human factor H (Sigma, St Louis, MO, USA). For liquid-phase absorptions, 10 nmol factor H were added per ml of optimally diluted antibody for 2 h at room temperature before incubating the tissue sections. For solid-phase absorptions, factor H was linked to Ultralink Biosupport Medium (Pierce, Rockford, IL, USA), the optimally diluted antibody was exposed to it for 2 h at room temperature, and the immune complexes removed by centrifugation.

Neither method resulted in a total abrogation of the signal, therefore we purified the antibody with an affinity chromatography column containing solid-phased factor H. Briefly, 1 mg factor H was covalently coupled to 250 mg UltraLink Biosupport Medium (Pierce) following the manufacturer's instructions. Rabbit anti-factor H serum (250 µl) was incubated with the resin for 16 h in 5 ml PBS at 4 °C. Afterwards, the resin was packed in a column and washed intensively with PBS. The bound antibody was eluted with 0.1 M citric acid, pH 3.3. The eluate was neutralized with 1 M sodium phosphate pH 8.0, and the buffer changed to PBS by ultracentrifugation in Centricon 50 columns (Amicon, Millipore Corporation, Bedford, MA, USA). Solid-phase absorption of this affinity-purified antibody resulted in a successful quenching of the staining.

Western blotting

Rat plasma (1 µl) from different rats was heated to 95 °C for 3 min in the presence of SDS and β-mercaptoethanol and loaded into the sample well. Purified human factor H (Sigma) was added in an adjacent lane for comparison. Samples were electrophoretically separated on a gradient

3–8% Tris–acetate gel (Novex, San Diego, CA, USA), and run at 100 V for 2 h. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a 0.2 µm nitrocellulose membrane at 30 V for 1.5 h. The membrane was blocked overnight with 5% non-fat dry milk in PBS, and incubated for 1 h in goat anti factor H (1:2000). The membrane was further exposed to peroxidase-bound rabbit anti-goat immunoglobulins (Sigma), 1:10 000 for another hour, and then peroxidase activity was revealed with the ECL+Plus chemiluminescence kit (Amersham, Arlington Heights, IL, USA) following the manufacturer's instructions.

Triple immunofluorescence and confocal microscopy

To characterize the cell type expressing factor H immunoreactivity, triple immunofluorescence with primary antibodies raised in three different species was used. After blocking non-specific binding with donkey normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), the paraffin sections were incubated in a mixture of the three primary antibodies. These included a guinea pig anti-bovine insulin 1:2000 (Jackson ImmunoResearch Labs), the affinity-purified rabbit anti-human factor H 1:200, and one of three anti-pancreatic peptide monoclonal antibodies. Monoclonal antibodies used in this study include: anti-somatostatin 1:10 000, anti-glucagon 1:1000, and anti-rat pancreatic polypeptide 1:500, all of them from CURE (UCLA, CA, USA). After an overnight incubation at 4 °C, the sections were incubated for 1 h in the second layer, which was a mixture of Cy5–donkey anti-guinea-pig (Jackson ImmunoResearch Labs), Bodipy–anti-mouse (Molecular Probes, Eugene, OR, USA), and biotinylated goat anti-rabbit (Dakopatts), all at a final concentration of 1:200. A third layer containing lysissamine rhodamine streptavidin 1:200 (Jackson ImmunoResearch Labs) was added for an additional hour. After thorough washes, the slides were mounted in SlowFade solution (Molecular Probes) and observed with a Zeiss Laser Scanning microscope 510, equipped with four lasers.

Electron microscopy

The pancreata of four additional rats were used for immunoelectron microscopy. Small pieces of the organ were fixed in 1% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4 °C for 2 h, dehydrated and embedded in the epoxy resin, TAAB-812 (TAAB Labs, England). Semithin (1 µm thick) sections were stained with methylene blue and used to select areas of interest or subjected to immunohistochemical staining as above.

Ultrathin sections (60–80 nm) were placed on nickel grids, etched for 5 min in 3% H₂O₂, and subjected to double immunogold staining as previously described (López *et al.* 1999). Briefly, the sections were incubated in

a mixture of both primary antibodies (anti-insulin and anti-factor H) at 4 °C overnight. On the following day, they were exposed to a second layer containing gold particles coupled to the appropriate secondary antibodies (E-Y Laboratories Inc., San Mateo, CA, USA) such that the larger gold particles (20 nm in diameter) recognized the insulin antibody, and the small particles (10 nm) the factor H antibody.

Following thorough rinses, the grids were stained for 15 min in 5% aqueous uranyl acetate and for 7 min in lead hydroxide. The sections were observed in a JEOL-1010 electron microscope.

RT-PCR

To validate the expression of complement factor H in the pancreas, an RT-PCR experiment was performed with specific primers. Since the sequence for the rat complement factor H gene is not available, primers were designed from the mouse gene (GenBank accession number M12660): sense (1877–1896) 5'-TTG-GAA-TTC-TCC-TGC-CAT-TC-3' and antisense (2644–2663) 5'-ACC-TTC-CAT-CTT-TGC-ACA-CC-3'. Total RNA was prepared from the liver and pancreas of two Balb/c mice, reverse transcribed using the SuperScript Preamplification System (Life Technologies, Gaithersburg, MD, USA), and PCR amplified with eLONGase enzyme mixture (Life Technologies) for 35 cycles, with an annealing temperature of 57 °C. The PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced by Lark Technologies (Houston, TX, USA). For southern blotting, the PCR products were transferred to a nitrocellulose membrane and an internal probe (2331–2350) 5'-TTG-CAA-CAG-ACC-AAC-TGG-AG-3' was end-labeled with standard procedures and used to confirm the specificity of the amplicons.

In situ hybridization

Detection of factor H mRNA in rat pancreas was performed using *in situ* hybridization as previously described (Martínez *et al.* 1996). The plasmid containing the pancreatic factor H PCR product was linearized with EcoRV and BamHI and used as a template to synthesize digoxigenin-labeled sense and antisense RNA riboprobes. Hybridization was performed in a moist chamber at 42 °C for 20 h in a 20 µl volume containing 2.5 ng probe/µl. After washes of increasing stringency, visualization of digoxigenin was performed using a digoxigenin detection kit (Boehringer Mannheim, Indianapolis, IN, USA). Controls included the use of the sense probe and digestion with RNase before hybridization.

Islet isolation, insulin secretion and RIAs

Pancreatic islets were isolated from 12 rats as previously described (Martínez *et al.* 1996). Briefly, the rats were

perfused through the heart with Hank's balanced salt solution (HBSS, Sigma) to remove blood from pancreatic vessels. A small cannula was inserted into the common biliary duct while the coledoco was sealed. A 20–25 ml volume of 0.4 mg/ml collagenase XI (Sigma) in HBSS was pumped through the cannula until the pancreas was fully inflated. The pancreas was removed and incubated at 37 °C for 20 min. After that, 20 ml ice-cold HBSS were added and the pancreas dissociated by agitation. After several washes, the islets were collected under a dissecting microscope and distributed into 24-well plates, with a minimum of 70 islets per well.

Freshly isolated islets were incubated in RPMI-1640 (Life Technologies) containing 5.6 mM glucose for 45 min at 37 °C. The supernatant was saved and the islets incubated in RPMI-1640 containing 20.6 mM glucose plus the potential (anti)secretagogues (AM and/or factor H) for another 45 min. The medium was again saved for future analysis and the islets extracted in ice-cold ethanol to measure cAMP contents. Insulin and cAMP were measured by RIA with commercial kits (Amersham), following the manufacturer's protocols.

Insulin release is expressed as a ratio between the insulin concentration in the medium containing high glucose and low glucose, to allow for variations in the number of secreting cells and/or their secretory efficiency. Graphs represent the mean and standard deviation of three wells per treatment. These experiments were performed three times with comparable results. Two-tailed Student's *t*-test was performed to determine statistical significance. *P* values <0.05 were considered statistically significant.

Results

Distribution of factor H immunoreactivity

The application of all the commercially available antibodies to paraffin-embedded tissue sections of rat pancreas resulted in a clear labeling of the pancreatic islets (Fig. 1A,B). The staining for factor H was always prominent in the islets of Langerhans with a clear granular pattern (Fig. 1B), but it could also be seen in some exocrine acini (Fig. 1C). When absorption controls were performed to confirm these observations, no diminution of the staining was seen. Interestingly though, attempts to pre-absorb the primary antibodies with purified factor H in liquid phase produced a paradoxical increase in staining intensity. To overcome these difficulties, we purified the commercial antibodies with a factor H affinity column and when the eluate was used for immunohistochemistry it showed the exact same pattern as the unprocessed serum (Fig. 1D). The purified antibody could now be pre-absorbed by solid-phase methods resulting in a total quenching of the staining (Fig. 1E). Nevertheless, the liquid-phase absorption attempts still produced the paradoxical reaction (Fig. 1F), possibly due to the building of

Figure 1 Distribution of factor H immunoreactivity in the rat pancreas as seen by immunohistochemical and *in situ* hybridization methods. Antibodies against factor H stained cells in the islets of Langerhans (A), which presented a granular pattern at higher magnification (B). Sporadically, some exocrine acini were also stained with the anti-factor H antibodies (C). Affinity-purified antibodies showed the same pattern of staining (D), which was totally abolished after solid-phase pre-absorption (E), but produced a paradoxical labeling increase after absorption in liquid phase (F). Serial sections showing that the antisense probe for factor H labeled mainly the pancreatic islets (G), while the sense probe did not produce any staining (H). The arrows point to the same islet of Langerhans in the consecutive sections. A, Bar=100 μ m; B, bar=5 μ m; C, bar=10 μ m; D–F, bar=50 μ m; G and H, bar=50 μ m.

large antibody–antigen complexes in the immunoreactive sites, as has been previously described for other epitopes (Larsson 1988).

To make sure our antibody was recognizing rat factor H, a Western blot experiment was run with rat

plasma (Fig. 2). The antibody identified a single band both in the lane of human purified factor H and in the lanes where rat plasma was loaded. A small difference in size was observed, with the rat band being slightly smaller than human factor H.

Figure 2 Western blot analysis of purified human factor H (50 ng) and rat plasma (1 µl) from three different animals (1–3). The antibody used here is the same used in the immunohistochemical analysis, goat anti-human factor H, at 1:2000 dilution. The apparent size of human factor H under reducing conditions is 150 kDa. The rat immunoreactive band is slightly smaller.

To identify the cell type responsible for the factor H immunoreactivity, multiple immunofluorescence followed by confocal microscopy was performed. In all cases, factor

H immunoreactivity was co-localized with insulin in the center of the pancreatic islets, whereas the peripheral cell types containing somatostatin, glucagon, or pancreatic polypeptide did not contain the factor H-like epitope (Fig. 3).

This observation was further confirmed by double immunogold staining under the electron microscope. Both insulin and factor H immunoreactivities were found in the secretory granules of the β cells and were absent from the other endocrine cell types (Fig. 4A). Interestingly, the small gold particles decorating the anti factor H antibody were preferentially located in the electron-lucent haloes of the secretory granules, whereas the larger gold particles labeling insulin were mostly found in the dense cores (Fig. 4B). Occasionally, some factor H immunoreactivity was found in the larger secretory granules of the exocrine cells (Fig. 4C), corroborating the light microscopic observations.

Figure 3 Triple immunofluorescence for insulin (purple), factor H (red), and other classical pancreatic hormones (green) in paraffin-embedded sections of rat pancreas. The fourth column represents a composite of the previous three images. In all cases factor H (FH) co-localizes with insulin (INS) in the central β cells and is absent from the peripheral cells which produce somatostatin (SST), glucagon (GLUC), or pancreatic polypeptide (PP). Confocal microscopy. Bar=20 µm.

Figure 4 Double immunoelectron microscopy for factor H (small gold particles, 10 nm in diameter) and insulin (large particles, 20 nm) in rat pancreas. Low magnification images (A) show that immunoreactive cells have the characteristic morphology of the β cells (β , arrows show the limits of the cell). A higher magnification of the secretory granules (B) shows that factor H immunoreactivity is predominantly found in the electron lucent haloes (small arrows) while insulin-associated gold particles are mainly in the dense granule cores (large arrows). The secretory granules of some exocrine cells were also labeled for factor H but not for insulin (C). A, Bar=2 μ m; B and C, bar=0.2 μ m.

Expression of factor H mRNA in the pancreas

The existence of immunoreactivity for a protein does not necessarily imply that such protein is expressed in that particular site. The immunoreactivity could be due to the presence of an unrelated epitope or the protein may have been captured by the cell from an external source. The second possibility was especially concerning since factor H circulates in the blood in high concentration and the islets are well irrigated. To investigate the intrinsic expression of factor H in the pancreas, an RT-PCR experiment was performed in mouse tissue. Figure 5 shows that a specific band for factor H was found after amplification of pancreatic cDNA. Sequencing of the band demonstrated 100% identity with the published sequence of mouse factor H (Kristensen & Tack 1986), further demonstrating the expression of factor H in the pancreas. In addition, *in situ* hybridization with an antisense riboprobe labeled mainly the pancreatic islets (Fig. 1G), whereas the sense probe did not produce any significant staining (Fig. 1H).

Regulation of insulin release

Addition of exogenous AM to isolated islets resulted in a significant decrease of insulin release (Fig. 6A) mediated through elevation of cAMP (Fig. 6B), as previously reported (Martínez *et al.* 1996). The addition of factor H in the presence of AM produced a dose-dependent further

reduction of insulin secretion (Fig. 6A) with concomitant elevations of cAMP (Fig. 6B). Factor H alone also showed some effect on lowering insulin release from the islets (Fig. 6A).

Discussion

In this study we show that the AM binding protein, complement factor H, is expressed in the pancreas and that it contributes to the regulation of insulin secretion.

The major source of complement factor H seems to be the liver, although a variety of other tissues and cell types have been shown to express this blood component (Friese *et al.* 1999). Here we show that the pancreas, and especially the β cells in the center of the islets of Langerhans, are another source for factor H. The specificity of the immunohistochemical staining pattern was confirmed by solid-phase absorption of the affinity-purified antibody and also by the paradoxical increase in intensity after liquid-phase absorption, since this phenomenon only happens when a specific antibody-antigen interaction takes place, augmenting staining via cascade cross-linking of immune complexes (Larsson 1988). Western blot analysis of rat plasma showed a single positive band with a size slightly smaller than human factor H, possibly due to species variations as reported for the AM

Figure 5 RT-PCR experiment performed on cDNA from mouse liver and pancreas. Specific primers for factor H produced the expected 839 bp band. Sequencing of the PCR products confirmed their identity with mouse factor H. Eth. Br., ethidium bromide.

binding protein by Elsasser *et al.* (1999). The specificity of the RT-PCR band obtained from mouse pancreatic extracts was also verified by base sequencing.

Factor H and insulin immunoreactivities were found in the same secretory granules, but interestingly, with a different suborganellar distribution. A comparable fact was reported for glicentin and glucagon-like peptides in the secretory granules of the neighboring α cells (Varndell *et al.* 1985). Whether this unusual localization has any physiological meaning is difficult to address and may just reflect a differential precipitation behavior during tissue fixation.

Physiologically, the production of factor H by the β cells seems to be related with the regulation of insulin release. In the presence of extrinsic AM, factor H induces a dose-dependent inhibition of insulin secretion accompanied by an elevation in cAMP. This observation is in agreement with previous experiments done in Rat2 cells, in which exogenous factor H augments AM induced cAMP production (Pío *et al.* 2001). These results suggest that factor H may act as a chaperone molecule which facilitates the binding of AM to its receptor. This has been suggested for other activating binding proteins such as insulin-like growth factor binding proteins (Hwa *et al.* 1999). Moreover, some membrane components have been shown to bind factor H (Pangburn *et al.* 1991, DiScipio *et al.* 1998, Malhotra *et al.* 1999), and we can envisage a mechanism by which factor H and AM bind in solution and then the attachment of factor H to its membrane binding site would bring AM in the proximity of its own receptor.

An intriguing observation is that factor H by itself is able to elicit a weak response from the isolated islets, whereas it

Figure 6 Physiological actions of factor H (FH) on insulin secretion. Treatments with AM and/or factor H resulted in dose-dependent decreases in insulin secretion (A) and concomitant elevations of cAMP in the isolated islets (B). Values for each treatment represent the mean and standard deviation of three independent wells. Insulin release is expressed as the ratio between the concentration in the high glucose medium divided by the amount in the low glucose one to allow for variations in the number of secreting cells and/or their secretory efficiency. All values were statistically different ($P < 0.05$) from the control (first bar).

did nothing in the Rat2 experiments (Pío *et al.* 2001). This apparent discrepancy may be due to differences in the exposure time of the assays. Incubations of 5 min are sufficient to evoke cAMP changes, whereas in the islet experiments the incubation time with the anti-secretagogues was 45 min. In that time a considerable amount of AM could be secreted from the islets, as can be demonstrated by adding a monoclonal anti-AM antibody (MoAbG6) which results in a great increase in insulin secretion over untreated controls (Martínez *et al.* 1996). At this time we can not exclude an AM-independent effect for factor H on insulin secretion and that they have an additive effect rather than acting through a protein–protein interaction. Nevertheless, the fact remains that factor H influences this important physiological phenomenon.

Other complement components have been indirectly implicated in the reduction of insulin release. For instance, C1q and C3 seem to be responsible for the insulin secretion suppression induced by serum from newly diagnosed type 2 diabetic patients (Conroy *et al.* 2000).

Another aspect of factor H physiology that may make it important for pancreatic integrity is its capacity to suppress complement-mediated cell lysis. It has been shown that some bacteria which are able to recruit factor H in their surface are less sensitive to lysis (Neeleman *et al.* 1999). The same mechanism is used by tumor cells that express factor H and escape immune surveillance through the inhibition of the complement system (Fedarko *et al.* 2000, Junnikkala *et al.* 2000). It could be possible that β cells are normally protected by secreting factor H, and that under conditions reducing expression of this inhibitory protein they may become more susceptible to complement-mediated lysis. This process may ultimately result in type 1 diabetes. The complement inhibitory role of factor H is more potent in the presence of AM (Pío *et al.* 2001) and, in the pancreas, AM is readily available. The protective role of AM against harmful immune reactions has been demonstrated in a mouse model that overexpresses AM in the cardiovascular system. The transgenic mice were less likely to die from lipopolysaccharide-induced shock than their wild-type littermates (Shindo *et al.* 1999). Whether this protection is mediated through factor H remains to be investigated.

Important variations have been found in the circulating concentrations of complement components in type 1 and 2 diabetic patients (Violettes *et al.* 1983, Sundsmo *et al.* 1985, Charlesworth *et al.* 1987, Titov *et al.* 1987, Ebeling *et al.* 1999), but so far factor H has not been studied in detail. Future studies focusing on this molecule may enlighten our understanding of the effects of factor H in metabolic diseases.

The finding of factor H immunoreactivity in the secretory granules of the exocrine acinar cells suggests that factor H is also a component of the pancreatic fluid and that it may play some role in intestinal physiology. Other

components of the complement system are synthesized and secreted in the exocrine pancreas. These include C3, C4, and factor B (Sumiyoshi *et al.* 1997).

As new regulatory molecules are added to the number of physiological switches of insulin release, the components of the glycemia homeostasis equation grow exponentially larger, adding new targets for pharmacological intervention in metabolic disorders such as diabetes and obesity.

Acknowledgements

We thank Dr F Urbano (Electron Microscopy Center, School of Medicine, Universidad Autónoma de Madrid) for the use of his facility. Monoclonal antibodies against somatostatin, glucagon and pancreatic polypeptide were kindly provided by Dr H C Wong (CURE/UCLA/DDC Antibody-RIA Core, NIH Grant DK41301). R Pío was supported by a training grant (98/9172) from Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain.

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Received 24 January 2001

Accepted 30 May 2001