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Peptidomic Profiling of Secreted Products from Pancreatic Islet Culture Results in a Higher Yield of Full-length Peptide Hormones than Found using Cell Lysis Procedures

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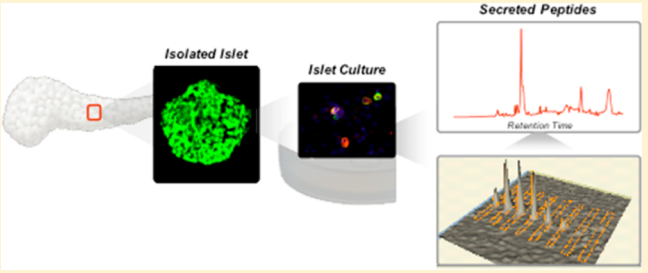
Supporting Information

ABSTRACT: Peptide Hormone Acquisition through Smart Sampling Technique-Mass Spectrometry (PHASST-MS) is a peptidomics platform that employs high resolution liquid chromatography−mass spectrometry (LC−MS) techniques to identify peptide hormones secreted from *in vitro* or *ex vivo* cultures enriched in endocrine cells. Application of the methodology to the study of murine pancreatic islets has permitted evaluation of the strengths and weaknesses of the approach, as well as comparison of our results with published islet studies that employed traditional cellular lysis procedures. We found that, while our PHASST-MS approach identified fewer peptides in total, we had greater representation of intact peptide hormones. The technique was further refined to improve coverage of hydrophilic as well as hydrophobic peptides and subsequently applied to human pancreatic islet cultures derived from normal donors or donors with type 2 diabetes. Interestingly, in addition to the expected islet hormones, we identified alpha-cell-derived bioactive GLP-1, consistent with recent reports of paracrine effects of this hormone on beta-cell function. We also identified many novel peptides derived from neurohormonal precursors and proteins related to the cell secretory system. Taken together, these results suggest the PHASST-MS strategy of focusing on cellular secreted products rather than the total tissue peptidome may improve the probability of discovering novel bioactive peptides and also has the potential to offer important new insights into the secretion and function of known hormones.



KEYWORDS: *peptidomics, FT-MS, secreted peptides, hormones, pancreatic islets*

INTRODUCTION

Endogenously secreted peptides have important biological actions as hormonal mediators, neurotransmitters and growth factors, and dysregulated peptide hormone signaling is implicated in the pathophysiology of a broad array of diseases.[1,2] Peptide hormones, which form the basis of the majority of approved peptide therapeutics, primarily target Class B G protein-coupled receptors (GPCRs), members of a transmembrane receptor superfamily widely considered the largest pharmaceutical target.[3−5] Until recently, challenges to the successful development of peptides as drugs have included physicochemical properties such as metabolic instability and poor bioavailability, a high cost of production, and an injectable route of delivery.[6] However, as naturally occurring biologics, peptides also demonstrate significant advantages over other compound classes. Peptide hormones have evolved to be highly potent and selective at their cognate receptors. In addition, peptide-based drugs generally display low toxicity and few adverse events. These attributes, coupled with technological advances in production, formulation and delivery, have led to a considerable revival of interest in pharmaceutical peptide discovery.[7−9]

Current estimates of novel peptide hormones yet to be identified are in the order of a few hundred, with 108 orphan GPCRs predicted to have peptide ligands.[10,11] Early peptide hormone discovery was generally empirical, based on phenotypic observations coupled with laborious bioassay-guided extraction and purification procedures. The study of peptides has since advanced considerably with the advent of contemporary mass spectrometry-based proteomic technologies that facilitate the cataloging of the protein content of any given cell or tissue from small quantities of biological material.[12,13] However, secreted peptides constitute only a very small fraction of the total peptide pool, are often present at low nanomolar concentrations and may require functional modifications for bioactivity. Therefore, standard proteomic methods involving trypsin digestion have severe limitations for the study of bioactive peptides that include low signal-to-noise due to sample complexity and the inability to comprehensively characterize post-translational processing. To minimize these hurdles to bioactive peptide discovery, we have developed Peptide Hormone Acquisition through Smart Sampling Technique-Mass Spectrometry (PHASST-MS), a peptidomics platform that leverages the sensitivity of high resolution mass spectrometry to analyze the complement of peptides within

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cultured endocrine cell secretions with minimal sample manipulation after MALDI TOF screening. Importantly, PHASST-MS is a "top down" proteomic approach[14] (i.e. avoiding exogenous protease digestion to generate smaller peptide fragments). Consequently, endogenous peptide processing and precursor cleavage sites are identified that may prove crucial for biological activity. A critical component of our strategy is the availability of *in vitro* or *ex vivo* endocrine models representative of *in vivo* physiology that allow us to probe the cellular secretome under conditions of pharmacologic or physiologic stimuli, and in the normal versus disease state. We have previously described the development of this technique using primary colonic crypt cultures.[15] In the present report, we describe further refinement and validation of these methods.

The islets of Langerhans in the pancreas play an important role in maintaining glucose homeostasis through secretion of peptide hormones such as glucagon and insulin from alpha and beta-cells, respectively.[16] Islet endocrine cells have been the subject of intense investigation since insulin was first used successfully to treat diabetes in the 1920s. Additional probing of beta-cell granules led to the discovery in 1987 of the hormone amylin, from which pramlintide, a marketed therapy for type 1 diabetes, has been derived.[17,18] As islet dysfunction is clearly important in the pathogenesis of both type 1 and type 2 diabetes, there continues to be strong interest in elucidating the underlying signaling mechanisms and mediators to develop novel strategies for therapeutic intervention. Thus, pancreatic islets have been extensively studied using a wide range of comparative and quantitative proteomic technologies.[19−21] There have been few peptidomic studies to date, and these have focused on probing either intact cells[22] or cellular lysates.[23] While these studies have greatly expanded our knowledge of the total islet proteome, identifying functionally important proteins and peptides from these complex data sets continues to be challenging. In our investigations, we have focused on a strategy that uses "top-down, label-free" mass spectrometry to analyze changes in cellular state at the secreted peptide level. Previously we have examined the secreted peptide profiles from an insulinoma derived beta-cell line.[24,25] However, cell lines, while readily studied, are poorly representative of normal physiology. In the present study, we apply the technique to *ex vivo* cultures of mouse and human pancreatic islets. Through peptidomic profiling of conditioned media from primary islet cultures rather than cellular lysates, we report many more identifications of known peptide hormones at various stages of post-translational processing than previous studies.

EXPERIMENTAL SECTION

Islet Isolation and Culture

All animal procedures were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, LLC in accordance with Animal Welfare Act guidelines. Murine pancreatic islets were harvested from anesthetized adult C57BL/6 mice using a modification of the method of Gotoh et al., 1990.[26] Briefly, following perfusion of the common bile duct with ice cold Liberase RI (Roche Applied Science) in M199 media, pancreata were excised and subjected to further enzymatic digestion for 18 min at 37 °C. Digested islets were immediately purified from acinar tissue using a density gradient of Histopaque 1077 (Sigma). Forty murine islets were collected using a dissecting microscope, digested to a single cell suspension with TrypLE (Gibco) and cultured on collagen 1 coated 24-well plates at a density of 70000 cells/mL/well in Pancreatic Islet Medium SBMI 06 (hCell Technology) supplemented with 6% FBS and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin) for 4 days at 37 °C in 5% CO_2. Freshly isolated human islets (5000−10000 IEQ at 90% purity) were obtained from the National Disease Research Interchange. Immediately upon arrival, human islets were washed and trypsinized to a single cell suspension. All subsequent handling and culture protocols were as described for mouse islets above.

Immunocytochemistry

Islet cultures were fixed in 10% neutral buffered formalin and stored at 4 °C before staining and analysis. Primary antibodies used were mouse monoclonal anti-GLP-1 (Amylin Pharmaceuticals, 1.03.03.;1:8000), and rabbit polyclonal anti-glucagon (Dako; 1:500) Secondary antibodies used were donkey anti-rabbit DyLight 594 (1:300) and donkey anti-mouse DyLight488 (1:300) (Jackson ImnunoResearch Laboratories). Nuclei were stained with 4,6-diamidino-2-phenylindole, dilactate (DAPI) (Molecular Probes, Carlsbad, CA).

Sample Collection and Preparation for MS Analysis

After 4−8 days in culture, murine and human islet cells were washed, and then stimulated by incubation with 10 μM forskolin for 1 h at 37 °C in RPMI 1640 medium supplemented with 17.5 mM glucose, 55 mM sodium pyruvate, and 15 mM HEPES. Murine islet media sample collection, solid phase extraction (SPE), desalting and concentration, and high resolution nanoflow LC−MS analysis were performed as described in Nikoulina et al. 2010,[15] but with the monoisotopic precursor selection feature disabled.

In addition to the SPE work up for hydrophobic peptides[15] which involved media samples being collected into an acidified aqueous organic solution at a final concentration of 25% acetonitrile, 2.5% formic acid, and 0.1% TFA, respectively, human islet media samples were also subjected to a complementary sample preparation technique favoring coverage of hydrophilic peptides. Media collected as described above was diluted to a final concentration of 12.5% acetonitrile and immediately subject to SPE. Concentration and desalting of human islet media/acetonitrile/TFA/formic acid samples by solid phase extraction was performed using a peptide MicroTrap (Thermo Fisher Scientific Inc.).[25] Hydrophobic peptides were eluted with 60% acetonitrile, 0.1% formic acid whereas hydrophilic peptides were eluted in 25% acetonitrile, 0.1% formic acid. In another procedural change, 10 μL aliquots eluted from SPE were collected and pooled based on their MALDI-TOF spectra (see below).

Pooled fractions in Sun autosampler vials (Sun-sri, Inc., part number 501 307) were brought to a final acetonitrile concentration of 30% for hydrophobic peptides and 5% for hydrophilic peptides in a final volume 50 μL prior to LC−MS.

MALDI-TOF MS

MALDI-TOF analysis was performed on each media sample post solid-phase extraction as a preliminary peptide profiling technique. Linear MALDI-TOF spectra were acquired as described in Nikoulina et al. 2010.[15] MALDI-TOF spectra were also acquired in reflector mode with an accelerating voltage of 20000, grid voltage of 66%, delay time of 375 ns, 100 shots per spectrum, and mass range of 1000−7000 Da, with a

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low mass gate of 1000 Da. Post-acquisition data analysis was performed using Data Explorer, v.4.0 (Applied Biosystems). MALDI spectra were baseline corrected and noise filtered/smoothed using a correlation factor of 0.7−1.0.

Microflow Liquid Chromatography/High Resolution Mass Spectrometry

Chromatography was performed using a Paradigm MS4B split flow HPLC system (Michrom Bioresources, Inc.) coupled to a 7T LTQ-FT Ultra hybrid linear ion trap (IT)-Fourier Transform Ion Cyclotron Resonance (FT ICR) mass spectrometer (Thermo Fisher Scientific Inc.) operated using Xcalibur 2.0 SR2/Tune Plus 2.2 operating system. The MS was fitted with an ADVANCE spray source (now commonly referred to as a "Captive Spray") (Michrom Bioresources Inc.).[27] This spray source allows the utilization of microliter flow rates, thus eliminating the need of trapping columns necessary for timely sample transfer in nanoflow techniques, but without sacrificing sensitivity and improving chromatography. Consequently, utilization of this source facilitates shorter analysis times and a more robust introduction of sample ions into the MS compared with the configuration previously employed.[15] To minimize adsorptive loss of peptides to HPLC hardware we employed PEEKSIL (75 μm ID) tubing between pumps, autosampler and column including a 50 μL PEEKSIL loop in the autosampler. Aliquots (50 μL) of sample were loaded on a Magic C18, 3u, 200A, C18AQ, 0.2 mm × 50 mm (Michrom) column. Mobile phase A was water containing 0.1% formic acid and B was acetonitrile containing 0.1% formic acid. The sample was loaded at 5 μL/min for 12 min and eluted with a linear gradient from 5% acetonitrile/0.1% formic acid to 60% acetonitrile/0.1% formic acid, or 40% acetonitrile/0.1% formic acid over 40 min at 2 μL/min for hydrophobic or hydrophilic peptides, respectively.

Full instrument settings for the LTQ-FT Ultra include a capillary temperature of 180 °C and a source voltage of 1.6 kV. A data-dependent selected ion monitoring (SIM) duty cycle was used, supplemented with IT MS3 and FT MS2 scans with dynamic exclusion enabled as described in detail in Nikoulina et al.[15] However, in the current study, the final scan event constituted either collisionally induced dissociation- (CID) or electron capture dissociation- (ECD) based FT MS2 fragmentation. In addition, the monoisotopic precursor selection feature was disabled. Automatic Gain Control (AGC) targets were 3 000 000 for FTMS full scans, 500 000 for FT SIM scans and 1 000 000 for FT MS2 scans. IT MSn spectra (Isolation width 2) and were collected in centroid mode, and FT full scan (m/z 400−1800), FT SIM and FT MS2 spectra (Isolation width 5) were collected in profile mode.

Peptide Identification

Peptide identification from high and low resolution MS/MS spectra was performed using a slightly modified strategy from that described in Nikoulina et al. 2010.[15] Ion trap and FT fragmentation experiments were analyzed by SEQUEST v.28 (revision 12) running on an eight-node Linux cluster with a Bioworks 3.3.1 SP1 (Thermo Fisher Scientific Inc.) interface. Searches were performed specifying monoisotopic precursor and fragment masses. Peak lists were generated using extract_msn version 4.0 specifying a group scan of 1, minimum ion threshold of 5, and intensity threshold of 100. Peptide mass tolerances were set at 2.5 and 1.0 Da on the fragments. Variable modifications specified were pyroglutamate (−17.0266 Da on Gln), methionine oxidation (+15.9949 Da), N-terminal acetylation (+42.0106 Da) and b ion fragmentation (−18.0106 Da) on the C terminus for MS3 data. Half cystine (−1.00782 Da on Cys) was specified as an additional variable modification corresponding to loss of a hydrogen atom on disulfide bridge formation. The March 24, 2009 release of the GenBankTM "non-redundant" protein databases were downloaded in FASTA format from the National Center for Biotechnology Information (NCBI) ftp site. An in-house Perl script was used to select sequences with mouse or human annotations from this file and, additionally, to remove all protein sequences containing nonspecific "X" residues. The edited FASTA-formatted file contained a total of 130 122 sequences for mouse and 206 387 sequences for human. No enzyme was specified in searching the databases for 600−7000-Da. For the mouse database, candidate peptides satisfying both criteria of Xcorr >1.5 (1+), >2.0 (2+), or >2.9 (≥3+) and peptide probability $p < 1 \times 10^{-5}$ were automatically accepted as reverse database searches[28] on the same files indicated a 0% false discovery rate using these parameters. For human peptides, which were identified from a larger database, the thresholds for SEQUEST identification were Xcorr >1.8 (+1), >2.5 (+2), >3.4 (≥+3) and $p < 1 \times 10^{-6}$.

FT fragmentation experiments were also analyzed using ProSightPC v2.0 RC 0.9.1 (Thermo Fisher Scientific Inc.) operated in high throughput mode.[29] Deconvolution of FT MS2 and ECD data and generation of monoisotopic mass lists were achieved using the Xtract algorithm. The precursor selection criteria were based on the peak of highest intensity, and multiple precursors were allowed with a relative precursor threshold of 10%. Other options were default, including a minimum signal/noise threshold of 3 and maximum charge of 40 on the precursor and fragments, respectively. Minimum fit and remainder thresholds were 40 and 20 for the precursor and 10 and 10 for the fragments, respectively. The minimum base peak fragmentation intensity was set at 1000 with the option to add the remainder afterward enabled. Deconvoluted FT MS2 and ECD experiments were filtered for a minimum intact mass of 1000 Da and 10 fragments and subject to a search tree involving (a) an absolute mass search and (b) a biomarker search against the same nonredundant mouse and human databases used in the SEQUEST searches (reformatted for ProSight). For absolute mass searches, the precursor mass and fragment masses (y and b ion for CID and c and z ion for ECD spectra, respectively) were specified as monoisotopic with a 10-kDa precursor window, a fragment tolerance of 10 ppm in ΔM mode, and the high priority PTM acetylation specified as a variable modification. Preliminary hit filtering involved a maximum of 25 sequences with a minimum of three matching fragments being returned. Searches resulting in an $E < 1 \times 10^{-4}$ were deemed a preliminary success and were automatically loaded into a peptide repository. Searches not satisfying this criterion were further analyzed in biomarker mode. Conditions were the same as the absolute mass mode except that ΔM mode was disabled, and a 10-ppm tolerance was set for the precursor. All raw files were searched in this way using forward and reverse databases. For the mouse searches it was found that an $E < 1 \times 10^{-7}$ corresponded to a 0% false discovery rate. For the larger human database, the threshold for an identification was $E < 1 \times 10^{-10}$. In absolute mass mode, preliminary peptide sequence identifications had to be reconciled with their intact masses because this mode of searching detects uniform mass shifts between calculated and observed mass resulting from extensions, truncations, substitutions, or modifications. This

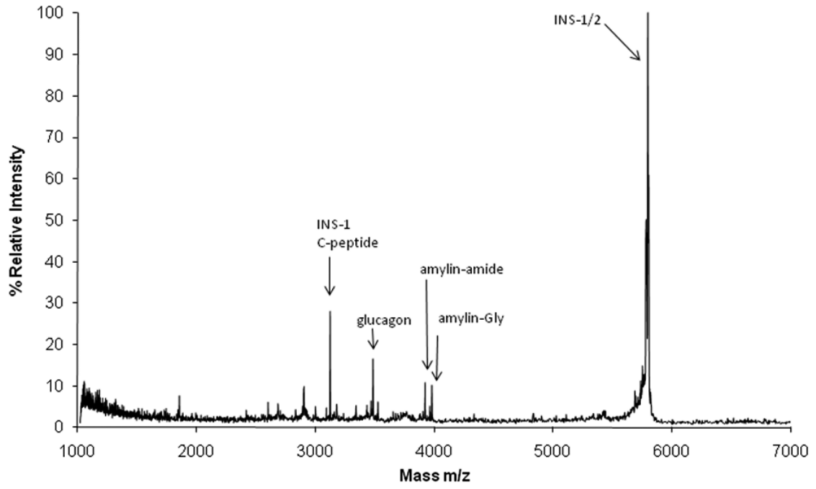

Figure 1. MALDI-TOF profile (Reflectron mode) of media from forskolin-stimulated murine islet cultures post solid phase extraction, pre-dilution, is dominated by major islet hormones.

was done through subsequent individual searches in biomarker, biomarker ΔM mode, or single protein searches with a proposed sequence.

Peptides identified satisfying the thresholds described above for either SEQUEST or ProSight searches were defined as satisfying Criterion 1. Peptides not satisfying these thresholds but with otherwise compelling evidence for a correct identification were also accepted (Criterion 2) with Supporting Information. This evidence included high resolution FT MS2 verification of consecutive b or y ion fragments or IT MS3 fragmentation confirming the sequence assignment of a gas phase b or y ion. Finally, peptide sequences were subjected to protein BLAST searches[30] against UniProt and NCBI databases, and redundant Swiss-Prot and single GenBank hyperlinked accession numbers are provided in the Supporting Information for those peptides with 100% homology. The GenBank accession number cross-referenced to the Swiss-Prot accession number was preferentially chosen.

RESULTS AND DISCUSSION

Murine Islets

In the present investigation we have conducted a nontargeted analysis of peptides released into murine islet culture media following cellular activation *in vitro*. By collecting only the secreted fraction, we aimed to reduce sample complexity and enhance the probability of detecting both known and novel peptides in their biologically active forms. In the current study, the major peaks identified in a preliminary MALDI-TOF survey of the secreted media represented the islet hormones, insulin, amylin and glucagon (Figure 1). These samples were then subjected to high resolution LC−MS analysis with the resulting peptide identifications shown in Table 1. More than 30 peptides from 16 gene products with an average molecular mass exceeding 3500 u (excluding mature insulin 1 and 2 which were identified solely by intact mass) were identified. These included 5 full length islet hormones, as well as several novel peptides from known prohormone precursors at various stages of post-translational processing. In addition, there were numerous peptides derived from proteins related to the neuroendocrine secretory system. The majority of these were from granins, a family of proteins localized in dense secretory vesicles that play an important role in granule biogenesis and hormone sequestration.[31] Interestingly, there is accumulating evidence that the granins are also precursor proteins that undergo proteolytic cleavage to produce small bioactive peptides such as secretoneurin, which was detected in this study.[32−34] Other peptides were derived from related proteins such as 7B2 and ProSAAS,[33] regulators of prohormone convertase, VGF,[33] reported to be involved in multiple endocrine functions, and annexin 1,[35] which is expressed in islets and may play a role in insulin biosynthesis and secretion.

All of the peptides identified in this PHASST-MS study (except ubiquitin and annexin A1) were derived from neurohormonal precursors or secretory proteins localized to the endocrine cell system. These included peptides derived from the C-terminal remnant region of from the prohormone precursor of Peptide YY,[23] such as a novel variant that we also recently found in endocrine cell enriched colonic crypts.[15] As many known peptides were successfully identified from these mouse islet cultures in their biologically active forms (referred to by name in Table 1), it will be of great interest to investigate the potential physiological functions of the novel secreted peptides that were also detected.

Human Islets

In preliminary studies to date, we have begun to use the PHASST-MS approach to profile human islets from 2 normal individuals and 1 individual clinically diagnosed with type 2 diabetes. Recognizing that the hydrophophilic peptide hormone somatostatin-14 was absent from our murine data set, we developed an alternative sample preparation strategy for more comprehensive peptide coverage in these valuable human samples. The strategy, developed by spike and recovery experiments using synthetic peptides added to RINm5F conditioned media (unpublished results), involved inclusion of a hydrophilic peptide biased workflow by reducing the amount of acetonitrile used in the media collection, SPE cleanup and autosampler vials. We also improved chromatographic robustness and resolution over our previously employed nanotrap configuration by utilizing the captive spray source with similar sensitivity to the nanoflow system, but using microliter flow rates to obviate the need for a trapping column. In addition, we employed ECD as a complementary fragmentation technique for peptide sequencing to identify more peptides. In total, 4 peptides were

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Table 1. Peptides Identified from Mouse Islet Culture Media[a]

gene and identified peptide	named peptides	ID method	MH^+ (calc)	delta (ppm)	z
Insulin 1					
GIVDQC@C@TSIC@SLYQLENYC@N+FVKQHLC@GPHLVEALYLVC@GERGFFYTPKS	Insulin 1	Intact mass	5800.687	3.3	6
EVEDPQVEQLELGGSPGDLQTLALEVARQ	C-peptide	SEQ_MS2	3120.570	−0.8	3
EVEDPQVEQLELGGSPGDLQTLAL		SEQ_MS2	2537.262	−0.8	3
EVEDPQVEQLELGGSPGDLQT		SEQ_MS2	2240.056	−0.7	2
Insulin 2					
GIVDQC@C@TSIC@SLYQLENYC@N+FVKQHLC@GSHLVEALYLVC@GERGFFYTPMS	Insulin 2	Intact mass	5793.612	4.3	5
EVEDPQVAQLELGGGPGAGDLQTLALEVAQQ	C-peptide	SEQ_MS2	3132.570	−1.8	3
EVEDPQVAQLELGGGPGAGDLQTLAL		SEQ_MS2	2577.304	−2.0	3
Amylin					
KC@NTATC@ATQRLANFLVRSSNNLGPVLPPTNVGSNTY[	amylin	SEQ_MS2	3918.970	1.5	4
KC@NTATC@ATQRLANFLVRSSNNLGPVLPPTNVGSNTYG		SEQ_MS2	3976.976	−5.8	4
Glucagon					
HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	GLP-1(1−37)	SEQ_MS2	4168.016	2.3	5
HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA	oxyntomodulin	PS	4448.170	2.8	6
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	glucagon	PS	3481.623	3.0	3
Pancreatic prohormone					
APLEPMYPGDYATPEQMAQYETQLRRYINTLTRPRYG	PP (1−37)	PS	4390.139	2.3	5
Peptide YY					
DVPAALFSKLLFTDDSDSENLPFRPEGLDQW		SEQ_MS2	3522.706	−0.5	3
DVPAALFSKLLFTDDSDSENLP		SEQ_MS2	2394.171	−0.7	2
Peptide YY, isoform CRA_a					
DVPAALFSKLLFTDDSDSENLPFRSRPEGLDQW		SEQ_MS2	3765.840	2.9	3
VGF nerve growth factor inducible precursor					
Q#AEATRQAAAQEERLADLASDLLLQYLLQGGARQ		SEQ_MS2	3680.899	−0.2	4
Neuroendocrine protein 7B2					
YSPRTPDRVSETDIQRLLHGVMEQLGIARPR		SEQ_MS2	3590.897	3.3	6
Neuroendocrine convertase 2					
Q#ELEEELDEAVERSLQSILRKN		SEQ_MS2	2611.321	3.1	3
Chromogranin-A					
AEDQELESLSAIEAELEKVAHQLQALRRG		SEQ_MS2	3233.676	2.8	5
AEDQELESLSAIEAELEKVAHQLQALRR[		SEQ_MS2	3175.671	2.0	3
AEDQELESLSAIEAELEKVAHQLQAL		SEQ_MS2	2864.452	3.9	4
AEDQELESLSAIEAELEKVAHQLQ		SEQ_MS2	2680.331	1.6	3
AEDQELESLSAIEAELEKVAHQL		SEQ_MS2	2552.273	1.2	3
Secretogranin-1 (Chromogranin-B)					
LGALFNPYFDPLQWKNSDFE	LE-20	SEQ_MS2	2401.150	−0.4	3
ADQRVLTAEEKKELENLAAMDLELQKIAEKFSQRG		SEQ_MS2	4002.096	1.8	5
Secretogranin-2					
IPVGSLKNEDTPNRQYLDEDMLLKVLEYLNQEQAEQGREHLA		SEQ_MS2	4896.447	3.0	6
IPVGSLKNEDTPNRQYLDEDMLLKVLEYLNQEQAEQGREHL		SEQ_MS2	4825.421	−1.0	5
Q#APYENLNDQELGEYLARMLVKYPELLNTNQL		SEQ_MS2	3762.868	−0.9	3
Q#APYENLNDQELGEYLARMLVKYPELLNTNQ		SEQ_MS2	3649.784	1.9	3
TNEIVEEQYTPQSLATLESVFQELGKLTGPSNQ	Secretoneurin	SEQ_MS2	3650.807	3.1	3
ProSAAS					
AVPRGEAAGAVQELARALAHLLEAERQE		SEQ_MS2	2955.576	1.1	4
Annexin A1					
A]MVSEFLKQARFLENQEQEYVQAVKSY	Ac(2−28)	SEQ_MS2	3277.620	2.3	3
A]MVSEFLKQARFLENQEQEYVQAVK	Ac(2−26)	SEQ_MS2	3027.524	1.5	3
Ubiquitin					
MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIF AGKQLED GRTLSDYNIQKESTLHLVLRLRGG		PS	8560.625	5.8	11

[a]Peptide modifications are indicated as follows:] *N*-Acetylation, # pyroglutamate, @ half cystine, [C-terminal amide, + hydrolysis (e.g. insulin 2 chains). For ID method, SEQ_MS2 or MS3 indicates that SEQUEST identified the peptide from an IT MS2 or MS3 experiment; PS indicates that ProSightPC was used with FT MS2 (CID) data unless ECD is indicated and Intact mass was used for the insulins only. Charge state of the precursor ion is indicated by z.

identified solely by ECD fragmentation and a further 13 peptide identifications were confirmed by their ECD spectra. The hydrophobic and hydrophilic peptide methodologies produce complementary MALDI-TOF profiles from human

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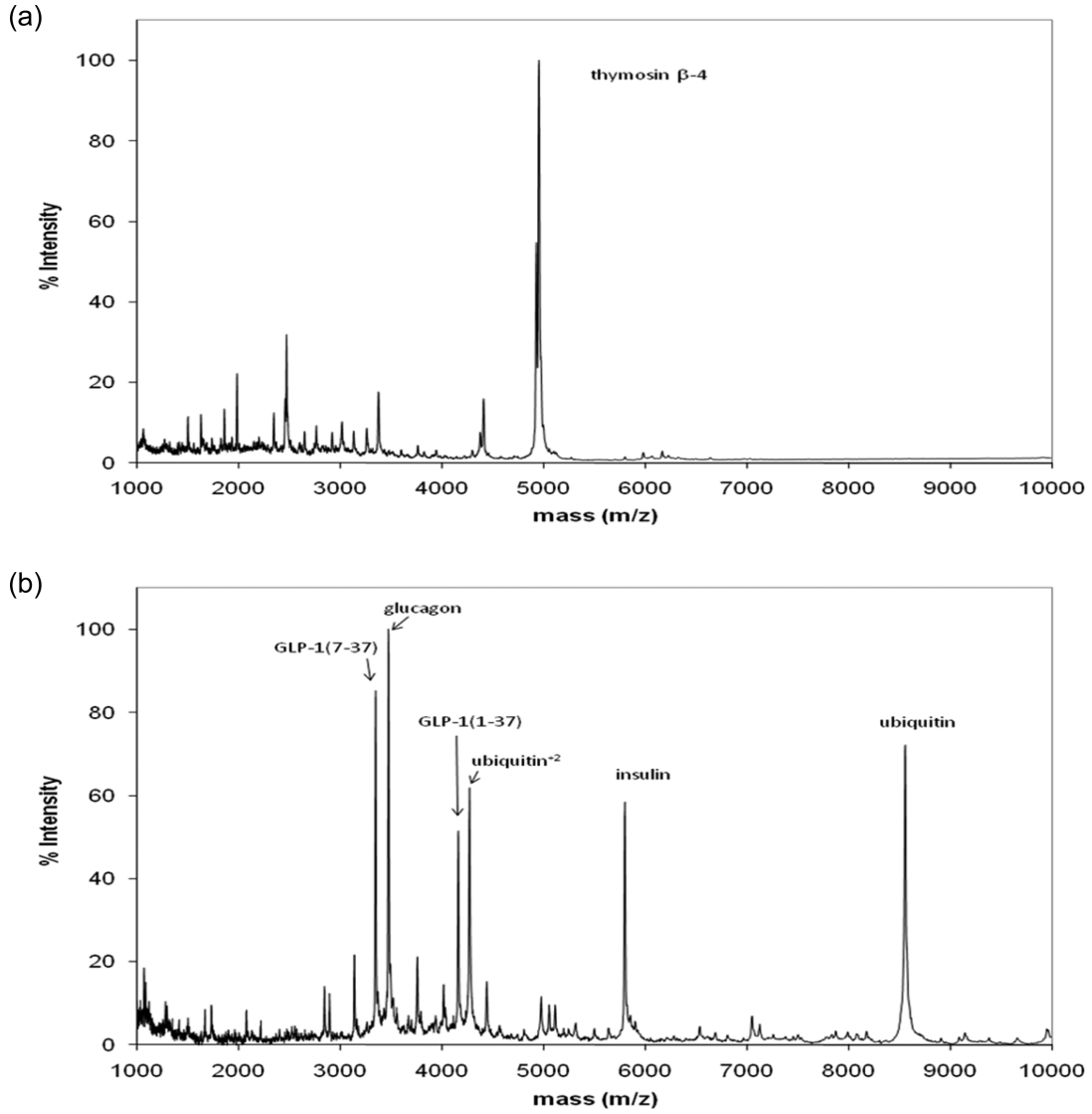

Figure 2. Comparison of MALDI-TOF profiles (Linear mode) of human islet conditioned media post solid phase extraction, pre-dilution for (a) hydrophilic and (b) hydrophobic peptides, respectively.

islet culture samples (Figure 2). By combining these with the above-mentioned improvements in chromatography and peptide sequencing capabilities, we demonstrate comprehensive coverage of all major secreted islet hormones at various stages of post-translational modification and processing. These ranged from small hydrophilic somatostatin-14 to large products of intermediary processing including the major proglucagon fragment (10 kDa) from alpha cells and C-peptide extended insulin (9.1 kDa) from the beta-cells (Table 2). A novel processed form of pancreatic polypeptide (PP) containing 3 residues of sequence considered to be in the signal region was identified in the islets from the individual with type 2 diabetes. Interestingly, SignalP versions 3[36] and 4[37] predict different cleavage sites in the PP precursor suggesting that the some ambiguity in processing of the prohormone may yet exist. Consistent with the murine data, we also identified many peptides derived from other neuroendocrine and secretory proteins such as the neuroendocrine regulatory peptide, NERP-2,[38] a bioactive peptide abundantly expressed in pancreatic islets that is derived from VGF,[39] a protein known to be involved in energy homeostasis and other metabolic functions. There were a few peptides derived from intracellular and cytoskeletal proteins that may reflect a greater level of cellular stress in the human islet cultures. However, there is also some evidence to suggest that these intracellular peptides may be secreted *via* nonclassical mechanisms and are biologically active.[40] Our current survey of human islets includes over 60 peptides from 24 gene products ranging from 1638 to 10837 Da in molecular weight (average around 4912 Da excluding mature insulin 1), +2 to +14 in charge (average around +6) with an average accuracy of around 4 ppm.

While this is a very limited sample set, we have made some interesting initial observations. MALDI-TOF profiling of the islet cultures from the two normal individuals exhibited qualitatively similar peptide hormone profiles whereas the individual with type 2 diabetes appeared to have elevated levels of pancreatic polypeptide, PP (1−36) when compared to the normal individuals (see Supporting Information). While analysis of additional human islet samples will be required to confirm potential qualitative or quantitative differences, an increase in PP (1−36) secretion in the individual with type 2 diabetes would be consistent with published reports suggesting

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Table 2. Peptides Identified from Human Islet Cultures[a]

gene and identified peptide	named peptides	ID method	MH^+ (calc)	deltaM (ppm)	z
Insulin 1					
GIVEQC@C@TSIC@SLYQLENYC@N +FVNQHLC@GSHLVEALYLVC@GERGFFYTPKT	Insulin 1	Intact mass	5804.645	10.2	5
EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ	C-peptide	SEQ_MS2	3019.522	−0.4	3
EAEDLQVGQVELGGGPGAGSLQPLA		SEQ_MS2	2392.199	−1.2	2
EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQC@C@TSIC@SLYQLENYC@ N+FVNQHLC@GSHLVEALYLVC@GERGFFYTPKT		PS	9089.346	8.1	7
Amylin					
KC@NTATC@ATQRLANFLVHSSNNFGAILSSTNVGSNTY[	amylin	SEQ_MS2	3901.871	7.0	4
Glucagon					
HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGRRDFPE EVAIVEELGRRHADGSFSDEMNTILDNLAARDFINWLIQTKITD	Major proglucagon fragment	PS	9965.907	8.3	9
RSLQDTEEKSRSFSASQADPLSDPDQMNEDKRHSQGTFTSDY SKYLDSRRAQDFVQWLMNTKRNRNNIA	glicentin	PS	8096.841	3.4	10
RSLQDTEEKSRSFSASQADPLSDPDQMNEDKRHSQGTFTSDYSKYLDSR RAQDFVQWLMNT		PS	7130.294	8.9	7
HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGRRDFPEEVAIVEELG		PS	5907.900	9.8	6
HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA	oxyntomodulin	PS	4448.170	9.2	6
HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	GLP-1(1−37)	SEQ_MS2	4168.016	4.4	5
RSLQDTEEKSRSFSASQADPLSDPDQMNED	GRPP	SEQ_MS2	3383.493	3.0	4
RSLQDTEEKSRSFSASQADPLSDPDQMNE		SEQ_MS2	3268.466	1.2	4
SLQDTEEKSRSFSASQADPLSDPDQMNED		SEQ_MS2	3227.392	0.0	3
HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	GLP-1(7−37)	SEQ_MS2	3354.675	1.0	4
HADGSFSDEMNTILDNLAARDFINWLIQTKITD	GLP-2	SEQ_MS2	3764.822	0.2	3
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	glucagon	PS	3481.623	9.5	4
EGTFTSDVSSYLEGQAAKEFIAWLVKGRG	GLP-1(9−37)	SEQ_MS3	3146.579	6.3	4
LQDTEEKSRSFSASQADPLSDPDQMNED		SEQ_MS2	3140.360	3.3	3
LQDTEEKSRSFSASQADPLSDPDQMNE		SEQ_MS2	3025.333	3.0	3
HSQGTFTSDYSKYLDS		SEQ_MS3	1835.808	3.4	3
Pancreatic prohormone					
APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY[	PP (1−36)	PS	4180.086	8.3	5
APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRYG	PP (1−37)	PS	4238.091	7.0	5
APLEPVYPGDNATPEQMAQYAADLRRYINMLTRP		PS	3861.905	0.0	4
AQGAPLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRYG		PS	4494.208	5.5	5
Somatostatin					
AGC@KNFFWKTFTSC@	Somatostatin-14	PS ECD	1637.724	1.8	3
APSDPRLRQFLQKSLAAAAGKQELAKYFLAELLSEPNQTEND ALEPEDLSQAAEQDEMRLELQ		PS	7054.5382	9.9	6
APSDPRLRQFLQKSLAAAAGKQELAKYFLAELLSEPNQTEND ALEPEDLSQAAEQDEMRLE		PS	6813.396	1.9	6
Neurosecretory protein VGF					
APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEVRGA		PS ECD	3951.995	0.0	6
NAPPEPVPPPRAAPAPTHVRSPQPPPPAPAPARDELPDWNEVL PPWDREEDEVYPPGPYHPFPNYIRPR		PS	7749.864	−2.0	8
NAPPEPVPPPRAAPAPTHVRSPQPPPPAPAPARDELPDWNEVL PPWDREEDEVYPPGPYHPFPNYIRP		PS	7593.763	5.5	8
Q#AEATRQAAAQEERLADLASDLLLQYLLQGGARQRGLG[	NERP-2	SEQ_MS2	4063.143	1.1	4
Q#AEATRQAAAQEERLADLASDLLLQYLLQGGARQRGLGG		SEQ_MS2	4121.149	−0.3	4
Q#AAAQEERLADLASDLLLQYLLQGGARQRGLGG		SEQ_MS2	3464.824	1.3	4
RAQEEAEAEERRLQEQEELENYIEHVLLRRP		PS	3862.943	1.2	6
AQEEAEAEERRLQEQEELENYIEHVLLRRP		PS	3706.842	6.8	5
SPQPPPPAPAPARDELPDWNEVLPPWDREEDEVYPPGPYHPFPNYIRPR		PS	5697.762	3.4	6
Chromogranin A					
GYPEEKKEEEGSANRRPEDQELESLSAIEAELEKVAHQLQALRR[	GR-44	PS	5061.563	4.2	8
GYPEEKKEEEGSANRRPEDQELESLSAIEAELEKVAHQLQALRRG		PS	5119.568	6.5	7
HSGFEDELSEVLENQSSQAELKEAVEEPSSKDVME		SEQ_MS3	3906.771	3.6	4
LEGQEEEEDNRDSSMKLSFRARAYGFRGPGPQL		PS ECD	3769.799	1.6	5
Q#AEGDSEGLSQGLVDREKGLSAEPGWQA		SEQ_MS3	2897.355	−1.2	3
WSKMDQLAKELTAE	WE-14	SEQ_MS2	1649.820	0.0	2
Secretogranin-1 (Chromogranin B)					
LGELFNPYYDPLQWKSSHFE	LE-20	SEQ_MS2	2470.171	0.4	3
Q#YDRVAQLDQLLHY	BAM-1745	SEQ_MS2	1744.865	−0.3	2

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Table 2. continued

gene and identified peptide	named peptides	ID method	MH^+ (calc)	deltaM (ppm)	z
Secretogranin-2					
FPVGPPKNDDTPNRQYWDEDLLMKVLEYLNQEKAEKGREHIA		SEQ_MS2	4983.473	4.4	6
TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ	Secretoneurin	SEQ_MS2	3677.818	2.8	3
ProSAAS					
SVPRGEAAGAVQELARALAHLLEAERQE		PS	2971.571	1.6	4
Heterogeneous nuclear ribonucleoprotein A1					
SSGPYGGGGQYFAKPRNQGGYGGSSSSSYGSGRRF		PS	3607.633	2.6	5
Microtubule-associated protein 4					
A]DLSLADALTEPSPDIEGEIKRDFIATLE		SEQ_MS3	3171.594	8.6	3
NEDD8					
MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMND EKTAADYKILGGSVLHLVLALRGG		PS	8555.67	7.013	11
Ubiquitin					
MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLED GRTLSDYNIQKESTLHLVLRLRGG		PS	8560.625	8.0	10
SH3 domain-binding glutamic acid-rich-like protein 3					
S]GLRVYSTSVTGSREIKSQQSEVTRILDGKRIQYQLVDISQDNALRDEMRALAGNP KATPPQIVNGDQYCGDYELFVEAVEQNTLQEFLKLA		PS	10343.242	12.8	11
10 kDa heat shock protein, mitochondrial					
A]GQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKG KGGEIQPVSVKVGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD		PS	10836.849	−0.1	14
26S proteasome complex subunit DSS1					
S]EKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDD FSNQLRAELEKHGYKMETS		PS	8184.538	1.2	7
40S ribosomal protein S28					
M]DTSRVQPIKLARVTKVLGRTGSQGQCTQVRVEFMDDTSRSIIRNVKGPVREGD VLTLLESEREARRLR		PS	7879.220	4.9	10
M]DTSRVQPIKLARVTKVLGRTGSQGQC&TQVRVEFMDD TSRSIIRNVKGPVREGDVLTLLESEREARRLR		PS	7998.224	7.8	12
Acyl-CoA-binding protein					
S]QAEFEKAAEEVRHLKTKPSDEEMLFIYGHYKQATVGDINTERPGMLDFTG KAKWDAWNELKGTSKEDAMKAYINKVEELKKKYGI		PS	9949.999	11.2	12
Alpha-1-antichymotrypsin					
RTIVRFNRPFLMIIVPTDTQNIFFMSKVTNPKQA		SEQ_MS2	4023.182	7.9	5
Annexin A1					
A]MVSEFLKQAWFIENEEQEYVQTVK		SEQ_MS2	3088.497	0.0	3
Thymosin beta-10					
A]DKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS		SEQ_MS2	4934.530	4.3	6
A]DKPDM*GEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS		SEQ_MS2	4950.525	3.3	6
Thymosin beta-4					
S]DKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES		PS	4961.494	10.1	6
Glial fibrillary acidic protein					
LVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE		PS ECD	3928.016	3.1	6

[a]Peptide modifications are indicated as follows:] *N*-Acetylation, # pyroglutamate, * oxidation, @, half cystine, & cysteinyl-cysteine, [C-terminal amide, + hydrolysis (e.g. insulin 2 chains) (See Table 1 for ID method).

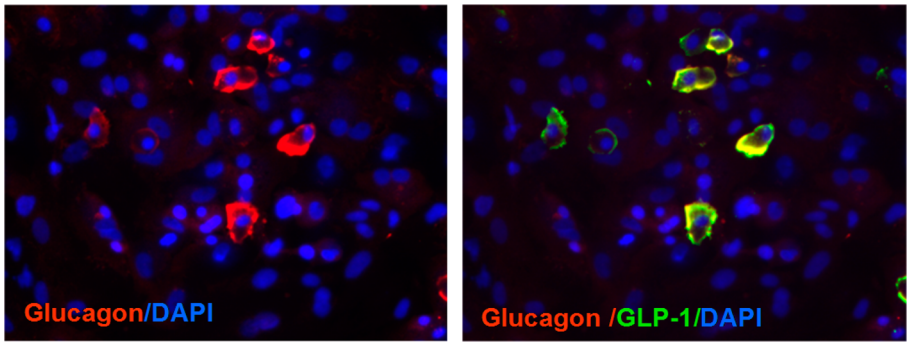

Figure 3. Immunostaining of human islet cells in culture indicate localization of glucagon (red), GLP-1 (green) and co-localization of GLP-1 and glucagon (yellow). Nuclei are stained with DAPI (blue).

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an association between elevated levels of this hormone and diabetes.[41−43]

We also report the first identification of human islet derived bioactive GLP-1 (7−37) by mass spectrometry techniques to our knowledge. Double-label immunocytochemistry shows colocalization of GLP-1(7−37) immunoreactivity with glucagon expression confirming its alpha-cell origin (Figure 3). GLP-1, an important glucoregulatory hormone, is usually secreted from intestinal L-cells following cleavage from the proglucagon precursor via the action of prohormone convertase, PC1/3.[44] Under normal conditions, adult pancreatic alpha-cells express PC2 which results in processing of the proglucagon precursor to produce glucagon. However, Kiliminik et al., 2010,[45] recently reported alpha-cell expression of PC1/3 in neonatal mice, and in mouse models of insulin resistance and prediabetes. In addition, studies of isolated rodent islets *in vitro* and *ex vivo* have demonstrated increased GLP-1 secretion following streptozotocin induced beta-cell toxicity,[46] or exposure to high glucose.[47] There is considerable evidence for direct actions of GLP-1 on promoting beta-cell survival and proliferation.[48] Thus, an increase in PC1/3 activity resulting in bioactive GLP-1 generation from the alpha-cell may reflect a paracrine mechanism by which local intercellular interactions contribute to the processes of pancreatic islet regeneration. Our data provide additional evidence for the intra-islet secretion of bioactive GLP-1 (7−37). These preliminary findings suggest that the PHASST-MS approach may be useful in identifying potentially important changes in peptide hormone secretion that may contribute to mechanisms underlying dysregulated glucose metabolism *in vivo*.

CONCLUSION

In summary, we report a peptidomic survey of primary mouse and human islet cell secreted products. Using our refined techniques, we identified all known islet hormones in their fully processed forms, as well as many novel peptides from neuroendocrine and secretory protein precursors. We also detected potential differences in known peptide hormone secretion that may reflect changes in physiological state *in vivo*. Thus, our data suggest that the PHASST-MS method for peptidomic analysis of the cellular secretome may represent a promising strategy for the discovery and profiling of functionally important endogenously secreted peptides.

ASSOCIATED CONTENT

Supporting Information

This article contains additional peptide identification details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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