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Human B-type natriuretic peptide is not degraded by meprin A

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ABSTRACT

B-type natriuretic peptide (BNP) combats cardiac stress by reducing blood pressure and ventricular fibrosis. Human BNP is inactivated by unknown cell surface proteases. N-terminal cleavage of mouse BNP by the renal protease meprin A was reported to increase inactivating degradation by a second protease named neprilysin. Since the sequence surrounding the meprin A cleavage site in BNP differs between species, we tested whether meprin A degrades human BNP. Using a recently developed proteolytic bioassay, the ability of various protease inhibitors to block the inactivation of BNP was measured. In rat kidney membranes, inhibitors of meprin A or neprilysin partially or completely blocked inactivation of rat BNP_{1-32} when added individually or in combination, respectively. In contrast, neither inhibitor alone or in combination prevented the inactivation of human BNP_{1-32} by human kidney membranes. Leupeptin, a serine protease inhibitor, totally blocked inactivation of human BNP by human membranes, substantially blocked the inactivation of rat BNP_{1-32} by human membranes, but had no effect on the inactivation of rat BNP_{1-32} by rat kidney membranes. Purified neprilysin reduced the bioactivity of rat BNP_{1-32} and human BNP. Digestion with both meprin and neprilysis caused the greatest reduction in rat BNP_{1-32} but had no effect on the bioactivity of human BNP_{1-32}. We conclude that meprin A does not degrade BNP in humans and should not be considered a pharmacologic target of the natriuretic peptide system.

1. Introduction

Cardiac natriuretic peptides are hormones/paracrine factors that regulate blood pressure by stimulating natriuresis, diuresis, vasorelaxation and antagonizing the renin/angiotensin system [1,2]. Atrial natriuretic peptide (ANP) is a 28 amino acid peptide that is stored in atrial granules. Human B-type natriuretic peptide (BNP) is a 32 amino acid peptide that is primarily released upon synthesis from the ventricles. Each peptide contains a 17-amino acid disulfide-bonded ring that is essential for biological activity [3]. Both ANP and BNP activate the transmembrane guanylyl cyclase natriuretic peptide receptor A (NPR-A) also called guanylyl cyclase A (GC-A). In addition, both peptides bind natriuretic peptide receptor-C (NPR-C), which clears them from the circulation via receptor mediated internalization and degradation [4].

Circulating levels of ANP and BNP are low under normal conditions but are dramatically elevated in response to cardiovascular stress [5–8]. Serum levels of both peptides are reduced by NPR-C-dependent intracellular degradation and by surface protease-dependent extracellular degradation. The protease most commonly associated with natriuretic peptide degradation is the ectoenzyme neprilysin—also called neutral endopeptidase (NEP) [EC 3.4.24.11]. This enzyme cleaves natriuretic peptides at multiple sites but the first and primary cleavage site within ANP and CNP is between the conserved cysteine and phenylalanine residues found in all natriuretic peptides [9,10]. This cleavage breaks the 17-amino acid ring and inactivates the peptides [3]. Enzymatic analysis indicates that CNP is the best substrate for NEP followed closely by ANP [9]. Interestingly, although BNP contains the same conserved cysteine and phenylalanine sequence (Fig. 1), NEP does not hydrolyze BNP at this position [9]. NEP does cleave BNP at other positions, but at low rates, which suggest that other enzymes mediate BNP degradation. Additional data supporting a NEP-independent BNP degradation pathway are the significantly elevated half-life of human BNP (~20 min) compared to ANP and CNP (~2 min) and data indicating that BNP levels are not elevated in mice lacking functional NEP [11].

In 2007, Pankow et al. [12] reported that BNP is inactivated through a sequential cleavage mechanism where the metalloprotease meprin A (EC 3.4.24.18) initially cleaves the amino terminus of the BNP peptide allowing subsequent inactivating cleavage of the ring by NEP. Subsequent studies by Boerrigter et al. [13] found

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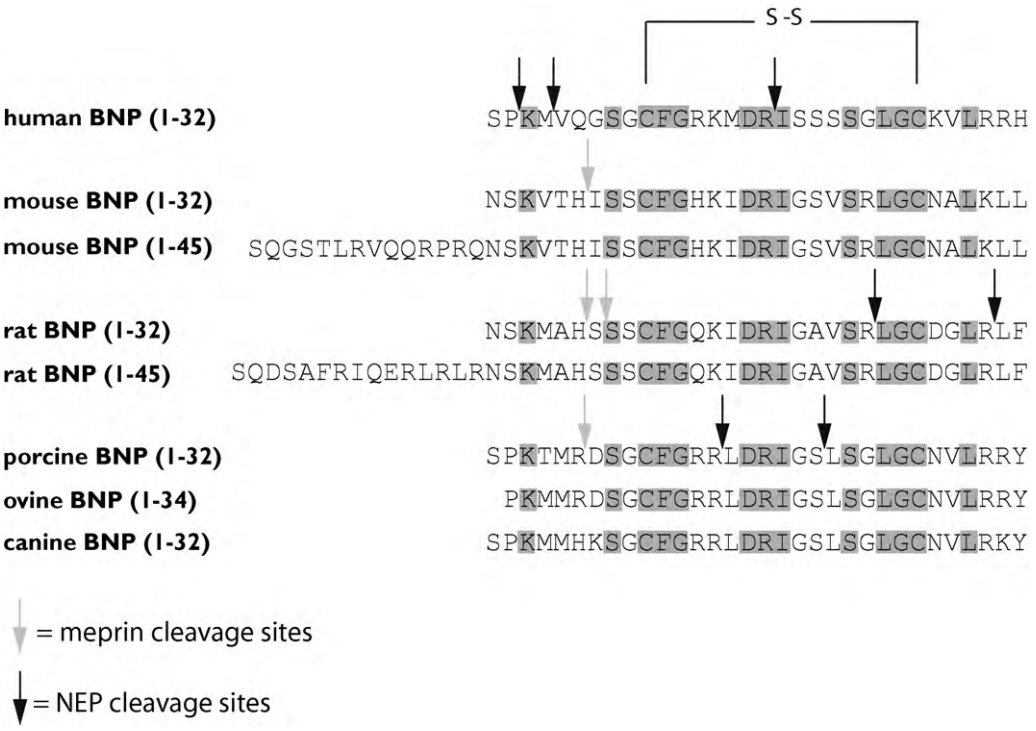

Fig. 1. Primary amino acid sequences of BNP from various species. The shaded areas indicate identical residues. The grey arrows indicate the location of the meprin-dependent cleavage. The black arrows indicate NEP cleavage sites.

that the meprin cleavage product, BNP_{8-32}, has reduced bioactivity in dogs. Here, we report that the proposed sequential meprin/NEP proteolytic degradation of BNP does not occur in humans, although it is operational in rats. We show that degradation of human BNP by human kidney membranes is unaffected by meprin or NEP inhibitors but is completely blocked by leupeptin, a general serine and cysteine protease inhibitor. These data indicate that the BNP degradation pathway has evolved and provide evidence of a novel pathway that is unique to humans. Importantly, it suggests that meprin A is not a reasonable pharmacologic target of the natriuretic peptide system.

2. Materials and methods

2.1. Reagents

Rat B-type natriuretic peptide 1–32 ($rBNP_{1-32}$), an amino-truncated form of the 45 residue natural rat form of BNP and human B-type natriuretic peptide ($hBNP_{1-32}$) were purchased from Phoenix Pharmaceuticals (Burlingame, CA). Protease inhibitors were from Roche Applied Science (Indianapolis, IN) (Complete Roche protease inhibitor cocktail tablet), MP Biomedicals, LLC (Solon, OH) (phosphoramidon), Sigma–Aldrich (St. Louis, MO) (actinonin) and Fisher Scientific (Pittsburgh, PA) (leupeptin and aprotinin). Purified recombinant human meprin A and neprilysin were from R&D Systems (Minneapolis, MN). Cyclic GMP radioimmunoassay kits were purchased from Perkin Elmer (Boston, MA).

2.2. Membranes

Human kidneys were obtained from the Tissue Procurement Program at the University of Minnesota. The investigation conforms with the principles outlined in the Declaration of Helsinki. Crude rat and human kidney membranes were prepared as described previously [14]. Briefly, tissues were weighed and then thawed in cold buffer (50 mM Tris–HCl, pH 7.5, 20% glycerol and 50 mM NaF). Membranes were homogenized, washed and stored at −80 °C until use. For each experiment, an aliquot of membranes was thawed and diluted to 2 mg/ml with ice-cold buffer.

2.3. Cells

Human embryonic kidney 293 cells stably expressing rat or human NPR-A were maintained as previously described [15].

2.4. Proteolysis bioassay

Proteolysis of natriuretic peptides was determined by a two-step process—proteolysis assay followed by a bioassay to measure the remaining peptide activity. Proteolysis was performed using 20 μl of crude membranes in a 0.1 ml total volume reaction and was carried out in a 37 °C water bath. Following incubation for the designated times, the proteolysis was stopped with 0.1 ml of 0.5N perchloric acid. Samples were then boiled for 10 min to further inactivate the proteolytic enzymes. Following neutralization with NaOH, an aliquot of the proteolysis sample was then mixed with DMEM containing 1 mM 1-methyl-3-isobutylxanthine (IBMX) and 25 mM HEPES, pH 7.4. This mixture was added to cells stably expressing rat or human GC-A to measure the bioactivity of the peptides produced after proteolysis. Cells were first incubated in serum-free medium for 4–6 h and then pretreated for 10 min at 37 °C in DMEM containing 25 mM HEPES, pH 7.4 and 1 mM IBMX. After pretreatment, the medium was replaced with the medium containing the proteolytic samples or increasing concentrations of untreated peptide. The cells were stimulated for 1 min (rat NPR-A) or 3 min (human NPR-A) and stopped by direct addition of 0.3 ml of 0.1N HCl. Cyclic GMP concentrations were estimated on a neutralized aliquot by radioimmunoassay. The amount (concentration) of peptide remaining in the proteolysis samples were estimated by fitting the amount of cGMP formed by those samples in the bioassay to a standard curve generated with known amounts

of (untreated) peptide. The resulting value for the "peptide only" sample in the proteolysis experiment was then set to 100%, and the calculated value for each proteolytic sample was graphed as a percentage of the starting "peptide only" value.

2.5. Analysis

GraphPad Prism was used for graphing the data. Data are presented as the mean ± standard error of the mean (SEM). Differences were considered significant when the p value was ≤ 0.05 using a paired Student's t-test.

3. Results

3.1. Meprin A and neprilysin do not inactivate human BNP

Various protease inhibitors were used to block degradation of human BNP_{1-32} (hBNP) by human kidney membranes. Incubation of hBNP with membranes for 20 min resulted in an 85% loss of the biologic activity of the peptide. Boiling the membranes prior to exposure to the peptide abolished the activity loss, consistent with activity reductions resulting from proteolysis. A compilation graph of several individual experiments is shown in Fig. 2. Neither phosphoramidon (an inhibitor of neprilysin) nor actinonin (an inhibitor of meprin) alone or in combination reduced human BNP inactivation by human kidney membranes. In contrast, the broad serine protease inhibitor, leupeptin, completely blocked activity losses.

3.2. Rat BNP is inactivated by meprin A

The scenario was completely different in the rat system. The NEP inhibitor significantly reduced the proteolytic inactivation of rat BNP_{1-32} by rat kidney membranes when added alone, and the inclusion of both the NEP and meprin inhibitors in the reaction significantly blocked the inactivation of the peptide (Fig. 3). In contrast to the human scenario (Fig. 2), leupeptin had no effect on the degradation of rat BNP by rat kidney membranes. The reason for the increase in activity when rat BNP was treated with boiled human kidney membranes or incubated in the presence of the three protease inhibitors is not known and was not investigated further since the main focus of these studies was to investigate the inactivation of human BNP. Overall, meprin contributes to the degradation of rat but not human BNP in kidney membranes and a leupeptin-sensitive protease is the major degrading enzyme in human but not rat membranes.

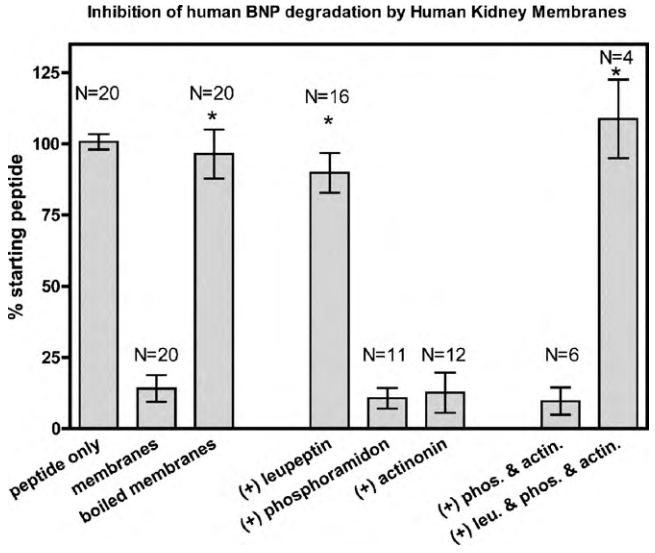

Fig. 2. Neither meprin nor NEP reduce the bioactivity of human BNP_{1-32}. Human BNP_{1-32} was incubated with 40 μg human kidney membranes for 20 min at 37 °C in the absence or presence of the indicated protease inhibitors and then the bioactivity of the resulting proteolyzed peptide was measured as described under Section 2. The values represent the mean ± SEM with the indicated number of replicates. *Significant difference between samples treated with membrane or membrane in the presence of the indicated inhibitor, $P < 0.0001$. The p value was not significant ($p < 0.05$) for all other treatments.

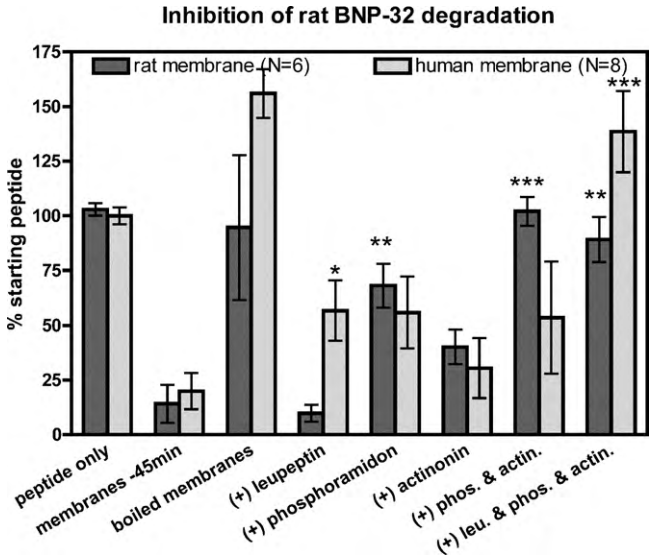

Fig. 3. Effect of various protease inhibitors on the inactivation of rat BNP_{1-32} by rat or human kidney membranes. Rat BNP_{1-32} was incubated with 40 μg rat or human kidney membranes for 45 min. Bioactivity was determined as described under Section 2. The values represent the mean ± SEM where $N = 6$ or 8 as indicated. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

3.3. BNP degradation is sequence and species dependent

To determine whether the difference in the inhibitor profiles of rat and human BNP was due to the species of the membrane or the amino acid sequence of BNP, we tested the ability of the human kidney membranes to degrade rat BNP_{1-32}. More than 75% of the bioactivity was lost when rat BNP_{1-32} was incubated with human kidney membranes for 45 min at 37 °C (Fig. 3). Both leupeptin and phosphoramidon blocked a portion of the activity loss, but actinonin in combination with phosphoramidon had little additional effect. Conversely human BNP_{1-32} was not a good substrate for rat kidney proteases (Fig. 4). After incubation at 37 °C for 45 min, only 48% of the initial human BNP bioactivity had been lost. Importantly, none of the protease inhibitor treatments resulted in a significant change in activity compared to human BNP treated with rat membrane only (Fig. 4).

3.4. Purified meprin and NEP do not inactivate human BNP

Finally, the ability of purified human meprin and NEP to degrade rat BNP_{1-32} and human BNP was tested (Fig. 5). Meprin A did not significantly reduce human BNP activity ($P = 0.27$) but did significantly reduce rat BNP_{1-32} activity. Purified NEP significantly reduced the bioactivity of both human and rat BNP ($P = 0.03$ and 0.002, respectively). Most importantly, incubation with purified meprin A and NEP caused the greatest reduction in rat BNP_{1-32} activity ($P < 0.0001$), whereas the addition of both purified enzymes failed to yield a significant reduction in the bioactivity of human BNP ($P = 0.28$). Increasing the incubation time to 1 h (versus 30 min) did not result in any additional loss of human BNP

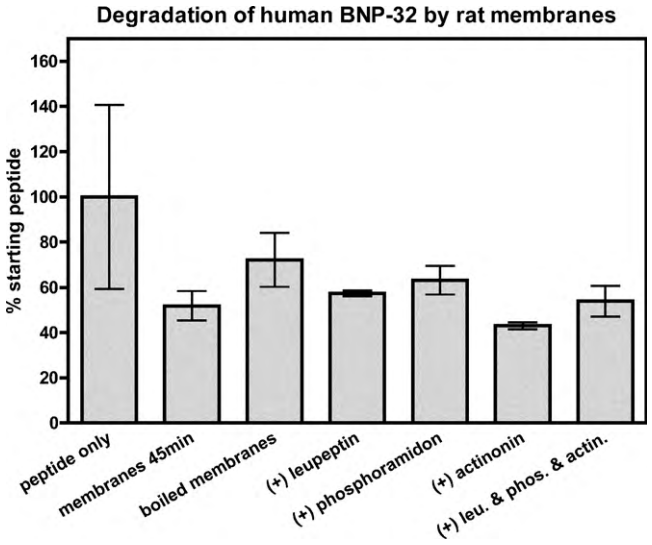

Fig. 4. Human BNP_{1-32} is resistant to degradation by rat kidney membranes. Human BNP_{1-32} was incubated with 40 μg of rat kidney membranes for 45 min. The amount of peptide remaining was determined as described under Section 2. The values represent the mean ± SEM where $N = 4$. None of the samples treated with membrane plus inhibitor were significantly different from membrane only treated samples.

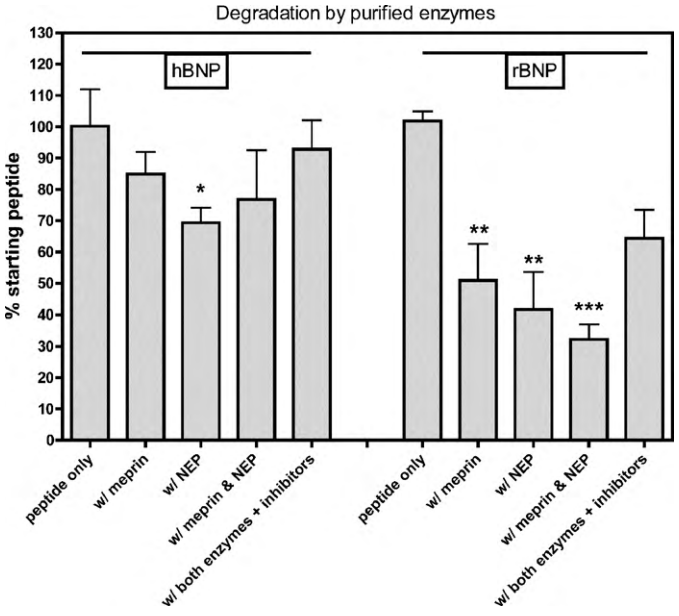

Fig. 5. Purified human meprin reduces rat but not human BNP activity. Rat or human BNP_{1-32} was incubated at 37 °C for 30 min with 40 ng recombinant human meprin, 50 ng of recombinant NEP or with both enzymes. Proteolysis was terminated with the addition of 0.5N perchloric acid. An aliquot of the proteolysis sample was then assayed as described under Section 2. The values represent the mean ± SEM where $N = 6$. Significance was determined by comparing the samples to peptide only, $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

activity nor did sequential addition of the enzymes (incubation with meprin for 30 min following by addition of NEP and additional incubation) (data not shown).

4. Discussion

Identification of the protease-dependent degradation mechanism of human BNP has important implications for the use of natriuretic peptide-based therapies such as nesiritide, which is approved for the treatment of heart failure in the USA and other countries. Data presented here clearly indicate that meprin A and neprilysin do not cleave human BNP via a sequential cleavage mechanism, thus bringing into question the utility of meprin A as a potential human drug target for the treatment of cardiovascular disease as has been suggested [12]. Nesiritide and synthetic BNP have the same amino acid sequence (human BNP_{1-32}) but are produced by different processes. Nesiritide is produced recombinantly in mammalian cells while the human BNP used in our studies was chemically synthesized. Thus, they should be degraded by the same mechanism. The differences in proteolysis reported between human and rat BNP also encourage caution when using animal models to examine the effect of BNP-based therapeutics on humans.

Inactivation of human BNP by human kidney membranes was blocked by boiling the membranes to denature cellular proteases or by incubation of the membranes with the general protease inhibitor leupeptin, consistent with activity losses resulting from proteolysis. However, neither the NEP inhibitor nor the meprin A inhibitor reduced bioactivity losses when added alone or together in the human system (Fig. 2). In contrast, the NEP inhibitor alone and in combination with the meprin inhibitor blocked the inactivation of rat BNP by rat membranes (Fig. 3). The lack of effectiveness of the inhibitors in the human assay system was not due to defective inhibitors because they worked as expected in the rat system. Because human BNP was not stabilized by inhibitors of NEP and meprin but rat BNP was protected from inactivation by these inhibitors in membranes from both species, it seems likely that susceptibility to degradation by these proteases is largely determined by the primary amino acid sequence of the BNP peptide. In contrast, the fact that leupeptin protected both human and rat BNP from inactivation in human but not rat membranes is consistent with the leupeptin-sensitive BNP protease only being present in human membranes. The identity of this protease is an important issue for future studies. Leupeptin is a broad range serine and cysteine protease inhibitor, which leaves a wide range of potential candidates for the BNP-degrading enzyme in human membranes. However, we have tested the serine proteases aprotinin and PMSF and neither had an effect on BNP inactivation (data not shown) suggesting the most likely candidate is a cysteine protease.

The different protease-dependent degradation mechanisms for rodent BNP versus human BNP is not unexpected for multiple reasons. First, the sequence of BNP varies greatly between species compared to the other natriuretic peptides. The same enzyme (rat NEP) that cleaves rat BNP_{1-32} at Arg^{23}-Leu^{24} and Arg^{30}-Leu^{31} [16] degrades porcine BNP at Arg^{14}-Leu^{15} and Ser^{20}-Leu^{21} [16,17]. Additionally, porcine NEP was shown to degrade human BNP at Pro^{2}-Lys^{3}, Met^{4}-Val^{5} and Arg^{17}-Ile^{18} [16]. Furthermore, human BNP is degraded by NEP at a much lower rate than CNP or ANP even though human BNP contains the primary cleavage site (Cys^{7}-Phe^{8}) found in ANP and CNP [9]. Thus, BNP appears to be a poor substrate for NEP and the cleavage sites are species dependent. Regarding the sequential degradation of BNP by meprin A and NEP, a comparison of the amino acid sequences of mouse, rat and human BNP illustrate that there is little sequence identity in the N-terminal region containing the meprin A cleavage site (Fig. 1). Murine BNP contains a His^{6} and Ile^{7} at the meprin A cleavage site [12], whereas human BNP contains very different residues (Gln^{6} and Gly^{7}) at these analogous positions. Consistent with this observation, we found that the species from which the protease is derived – rat or human – affects whether or not BNP is cleaved. Similarly, Norman et al. [16] found that NEP primarily cleaves human BNP between Arg^{17}-Ile^{18}, and although this same sequence is present in human ANP and in porcine BNP, rat NEP does not cleave these peptides at this position. Together with data

indicating differences in substrate specificity between human and mouse meprin A [18,19], these results support different degradation mechanisms for human and rodent BNP. Thus, identification of the cellular protease-dependent degradation of BNP in humans will have to come from direct analysis of human BNP degradation by human enzymes and not from animal models.

Finally, it should be noted that the most abundant form of BNP in rodent serum is BNP_{1-45}. The studies by Pankow and colleagues and those reported here were conducted using truncated forms of mouse or rat BNP (BNP_{1-32}). The importance of the meprin/neprilysin degradation pathway on the full-length rodent peptides remains unknown.

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