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Hypothesis

Consensus sequence for processing of peptide precursors at monobasic sites

Lakshmi Devi

Department of Pharmacology, New York University Medical Center, NY 10016, USA

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Many regulatory peptide precursors undergo post-translational processing at mono- and/or dibasic residues. Comparison of amino acids around the monobasic cleavage sites suggests that these cleavages follow certain sequence motifs and can be described as the rules that govern monobasic cleavages: (i) a basic amino acid is present at either 3, 5, or 7 amino acids N-terminal to the cleavage site, (ii) hydrophobic aliphatic amino acids (leucine, isoleucine, valine, or methionine) are never present in the position C-terminal to the monobasic amino acid at the cleavage site, (iii) a cysteine is never present in the vicinity of the cleavage site, and (iv) an aromatic amino acid is never present at the position N-terminal to the monobasic amino acid at the cleavage site. In addition to these rules, the monobasic cleavages follow certain tendencies: (i) the amino acid at the cleavage site tends to be predominantly arginine, (ii) the amino acid at the position C-terminal to the cleavage site tends to be serine, alanine or glycine in more than 60% of the cases, (iii) the amino acid at either 3, 5, or 7 position N-terminal to the cleavage site tends to be arginine, (iv) aromatic amino acids are rare at the position C-terminal to the monobasic amino acid at the cleavage site, and (v) aliphatic amino acids tend to be in the two positions N-terminal to and the two positions C-terminal to the cleavage site, except as noted above. When compared with a large number of sequences containing single basic amino acids, these rules and tendencies are capable of not only correctly predicting the processing sites, but also are capable of excluding most of the single basic sequences that are known to be uncleaved. Many of these rules can also be applied to correctly predict the dibasic and multibasic cleavage sites suggesting that the rules and tendencies could govern endoproteolytic processing at the monobasic, dibasic and multibasic sites.

Neuropeptide biosynthesis; Precursor processing; Peptide hormone; Growth factor

Peptide hormones and neurotransmitters are thought to be synthesized as larger precursors that undergo post-translational modifications and proteolytic cleavages to produce bioactive peptides. The regulatory peptides are flanked by either a dibasic or a monobasic processing site. Maturation of these bioactive peptides occurs by sequential action of trypsin-like and carboxypeptidase-B-like enzymes [1]. Although until recently only a few prohormones were known to be processed at monobasic cleavage sites, at present a large number of monobasic cleavages are found throughout the animal kingdom [2–6]. Table I shows the list of precursors and the amino acid sequence around the monobasic cleavage site.

Typically, fewer than 10% of the single basic sequences within the peptide precursor serve as monobasic cleavage sites. In contrast, the majority of the dibasic sequences within a peptide precursor usually are cleaved. Monobasic and dibasic cleavage sites also differ in the predicted secondary structure surrounding these sites. Rholam et al. [7] proposed that the dibasic processing sequences are located in or adjacent to regions with high β-turn probability [7]. A similar analysis on 20 different monobasic cleavage sequences showed that although some of the sequences were predicted to be in or around high β-turn probability, many were not (data not shown). In the case of the cleavage sites that contained proline either at the N- or at the C-terminus of the processing site, the cleavage site was situated in or near an α-helix. In the case of the sites that did not contain proline, there was no predicted conformational feature that was apparent. This could be due to the inability of Chou and Fasman analyses to accurately predict the secondary structure [8].

In order to ascertain if monobasic processing follows certain consensus sequences, Schwartz [2] compared the amino acid sequences around a number of monobasic cleavage sites and postulated that the monobasic processing sites can be classified as (i) 'proline-directed' and (ii) 'non-proline directed'. By a similar analysis of the sequences around the monobasic cleavage site, Benoit et al. [3] hypothesized that three principles govern the monobasic cleavages. They are: (i) the monobasic cleavage occurs virtually always at the carboxyl-terminal end of an arginine; (ii) a leucine or several alanines are virtually always present in the two amino acids immediately N-terminal to and the two

Correspondence address: L. Devi, Department of Pharmacology, New York Univ. Medical Center, 550 First Ave, New York, NY 10016, USA

amino acids C-terminal to the monobasic cleavage; (iii) three or five or seven amino acids before the arginine, a second arginine is present; sometimes a histidine (instead of an arginine) is found at this site. The principles proposed by Benoit et al. were based on analysis of 16 single basic sequences. Currently there are approximately 110 sequences that are known to be processed at proline-containing and non-proline-containing monobasic sites (including the same peptide in different species, and all copies of a peptide when present in multiple copies, as in the case of the caerulein precursor). The comparison of amino acids around all known monobasic cleavage sites shows that all three of the previously proposed principles are not entirely valid. However, additional rules are apparent from this analysis. These observations from the sequences around the monobasic cleavage sites can be encapsulated as four rules that govern monobasic cleavages.

Rule 1. A basic amino acid (Arg, Lys, His) is always present either at the −3, −5, or the −7 position (3, 5, or 7 amino acids N-terminal to the cleavage site).

Rule 2. Aliphatic amino acids (Leu, Ile, Val, Met) are never present at the +1 position (one amino acid C-terminal to the cleavage site).

Rule 3. A Cys is never present in the vicinity of the cleavage site, i.e. from 7 amino acid N-terminal to and 3 amino acids C-terminal to the cleavage site.

Rule 4. Aromatic amino acids (Phe, Tyr, Trp) are never present at −1 position (one amino acid N-terminal to the cleavage site).

In addition, monobasic cleavages follow certain tendencies. They are:

Tendency 1. The monobasic amino acid at the cleavage site is usually an Arg, occasionally a Lys, and never a His.

Tendency 2. Short side chain amino acids, predominantly Ser, Ala, or Gly tend to be at the +1 position (in approximately 70% of the cases).

Tendency 3. The basic amino acid at the −3, −5, or the −7 position tends to be Arg (in approximately 75% of the cases).

Tendency 4. Aromatic amino acids (Trp, Phe, Tyr) are rare in the +1 position.

Tendency 5. Aliphatic amino acids (Leu, Ile, Val, Met) and small side chain amino acids (Ser, Ala, Gly) often occur in the −2 position and in the +2 to +8 positions.

Schwartz [2] hypothesized that the proline-containing monobasic cleavages are distinct from the non-proline containing cleavages. The consensus site for proline-directed cleavages is simply the presence of a proline in the −1 or the +1 position (but never at both positions). It is interesting to note that in mammals the rules and tendencies predicted for non-proline-containing monobasic cleavage sequences also fit the proline-containing sequences (Table I).

The only known exception to rule 1 is peptide A of *Aplysia* [9]. Peptide A precursor contains a single Lys at the −6 position and none at the −3, −5 or the −7 positions. Although the cleavage to release peptide A is known to occur in the atrial gland, an identical cleavage site in the egg laying hormone (ELH) precursor is not cleaved in the bag cells [9]. Benoit et al. [3] proposed that an Arg or a His at either the −3, −5, or the −7 position is a necessary requirement for monobasic cleavages. We find that Arg is found predominantly (87%) at the −3, −5 or the −7 position (Tables I and II). In the remaining cases either a Lys or a His is found in one of these positions.

In order for a consensus site to be useful in predicting which single basic residue becomes a monobasic processing site, it is necessary to examine all single basic sites present in the regulatory peptides and proteins in the secretory granules. When the rules were applied to all the single lysines and arginines in approximately 90 bioactive peptides that are thought to be uncleaved, 90% of the cases did not fit the rules. Among the remaining 10%, very few fit the tendencies in addition to the rules. When the rules and tendencies were applied to all the single arginines in about 20 proteins that are contained in the secretory vesicles, in more than 95% of the cases the single arginines did not fit the rules and tendencies. It is possible that those sites that fit both the rules and tendencies are processed at that site. Alternatively, secondary and tertiary structure of the proteins may contribute to the ability of these sites to be processed. However, the fact that the rules and tendencies can exclude >90% of the single basic residues that are known to be largely uncleaved illustrates the value of these rules in predicting potential cleavage sites.

The rules are supported by evidence from naturally occurring mutations, both through evolution and within individuals. An example of mutation through evolution is that of pancreatic polypeptide [10]. Compared to the human pancreatic polypeptide precursor the primary structure of the guinea pig pancreatic polypeptide is missing one of the basic residues from a dibasic cleavage site [10] (Fig. 1). The resulting monobasic residue fits all the rules and is also processed in vivo [10]. Another example of mutation through evolution is that of β-endorphin [11]. Spiny dogfish β-End contains two insertions; a Lys between positions 5 and 6 and a Trp between positions 6 and 7 of human β-End (Fig. 1). As a result of these variations, the single

Table I

Partial sequences of peptide precursors with monobasic cleavage sites

-8	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8	Ref
-Arg-	-Arg-	-Gln-	-Phe-	-Lys-	-Val-	-Val-	-Thr-	-ARG-	-Ser-	-Gln-	-Glu-	-Asp-	-Pro-	-Asn-	-Ala-	-Tyr-	DynB[31]
-Asp-	-Glu-	-Met-	-Arg-	-Leu-	-Glu-	-Leu-	-Gln-	-ARG-	-Ser-	-Ala-	-Asn-	-Ser-	-Asn-	-Pro-	-Ala-	-Met-	SS28[32]
-Pro-	-Leu-	-Lys-	-Pro-	-Ala-	-Lys-	-Ser-	-Ala-	-ARG-	-Ser-	-Val-	-Arg-	-Ala-	-Gln-	-Arg-	-His-	-Thr-	IGF1[33]
-Ile-	-Phe-	-Gln-	-Val-	-Leu-	-Arg-	-Gly-	-Val-	-ARG-	-Ser-	-Pro-	-Lys-	-Thr-	-Met-	-Arg-	-Asp-	-Ser-	BNP [34]
-Asp-	-Lys-	-Arg-	-Phe-	-Met-	-Arg-	-Phe-	-Gly-	-LYS-	-Ser-	-Leu-	-Gly-	-Thr-	-Asp-	-Asp-	-Val-	-Asp-	FMRF[35]
-Pro-	-Arg-	-Leu-	-Arg-	-Gln-	-Phe-	-Leu-	-Gln-	-LYS-	-Ser-	-Leu-	-Ala-	-Ala-	-Ala-	-Thr-	-Gly-	-Lys-	Ant [36]
-Met-	-Leu-	-Thr-	-Arg-	-Pro-	-Arg-	-Tyr-	-Gly-	-LYS-	-Ser-	-Ala-	-Glu-	-Glu-	-Asp-	-Ala-	-Leu-	-Gly-	PanP[10]
-Glu-	-Glu-	-Ala-	-Pro-	-Arg-	-Arg-	-Gln-	-Leu-	-ARG-	-Ala-	-Val-	-Leu-	-Arg-	-Pro-	-Asp-	-Ser-	-Glu-	CC58[37]
-Gly-	-Ala-	-Val-	-Tyr-	-Gln-	-Arg-	-Asp-	-Leu-	-ARG-	-Ala-	-Pro-	-Arg-	-Leu-	-Arg-	-Phe-	-Tyr-	-Ser-	ELH [9]
-Gly-	-Val-	-Gly-	-Phe-	-Pro-	-Arg-	-Arg-	-Val-	-ARG-	-Ala-	-Asn-	-Asp-	-Arg-	-Ser-	-Asn-	-Ala-	-Thr-	VNII[38]
-Glu-	-Ser-	-Asn-	-Gln-	-Glu-	-Arg-	-Gly-	-Ala-	-ARG-	-Ala-	-Arg-	-Leu-	-Gly-	-Arg-	-Gln-	-Val-	-Asp-	HGRH[39]
-Glu-	-Asp-	-Ala-	-His-	-Ala-	-Asp-	-Leu-	-Glu-	-ARG-	-Ala-	-Ala-	-Ser-	-Gly-	-Gly-	-Pro-	-Leu-	-Leu-	SS1 [36]
-Gly-	-Phe-	-Met-	-Lys-	-Ser-	-Trp-	-Asp-	-Glu-	-ARG-	-Gly-	-Gln-	-Lys-	-Pro-	-Leu-	-Leu-	-Thr-	-Leu-	β-En[11]
-Asp-	-Asp-	-Val-	-Asn-	-Glu-	-Arg-	-Asp-	-Val-	-ARG-	-Gly-	-Phe-	-Gly-	-Ser-	-Phe-	-Leu-	-Gly-	-Lys-	Caer[40]
-Glu-	-Asp-	-Leu-	-Asp-	-Glu-	-Arg-	-Glu-	-Val-	-ARG-	-Gly-	-Ile-	-Gly-	-Lys-	-Phe-	-Leu-	-His-	-Ser-	Mega[41]
-Asn-	-Asp-	-Glu-	-Val-	-Glu-	-Arg-	-Tyr-	-Val-	-ARG-	-Gly-	-Trp-	-Ala-	-Ser-	-Lys-	-Ile-	-Gly-	-Gln-	Levi[6]
-Gln-	-Glu-	-Gln-	-Arg-	-Ser-	-Arg-	-Phe-	-Asn-	-ARG-	-His-	-Leu-	-Asp-	-Arg-	-Val-	-Trp-	-Ala-	-Glu-	RGRH[39]
-Arg-	-Asp-	-Leu-	-Arg-	-Trp-	-Trp-	-Glu-	-Leu-	-ARG-	-His-	-Ala-	-Gly-	-Tyr-	-Asp-	-Gln-	-Gly-	-His-	EGF [42]
-Tyr-	-Tyr-	-Asp-	-Val-	-Ser-	-Arg-	-Asn-	-Ala-	-ARG-	-His-	-Ala-	-Asp-	-Gly-	-Val-	-Phe-	-Thr-	-Ser-	PHM [43]
-Gly-	-Val-	-Arg-	-Ser-	-Pro-	-Lys-	-Thr-	-Met-	-ARG-	-Asp-	-Ser-	-Gly-	-Cys-	-Phe-	-Gly-	-Arg-	-Arg-	BNP [34]
-Cys-	-Ala-	-Thr-	-Pro-	-Ala-	-Lys-	-Ser-	-Glu-	-ARG-	-Asp-	-Val-	-Ser-	-Thr-	-Pro-	-Pro-	-Thr-	-Val-	IGII[44]
-Asp-	-Pro-	-Ser-	-His-	-Arg-	-Ile-	-Ser-	-Asp-	-ARG-	-Asp-	-Tyr-	-Met-	-Gly-	-Trp-	-Met-	-Asp-	-Phe-	CC8 [37]
-Gly-	-Glu-	-Asp-	-Gly-	-His-	-His-	-Leu-	-Asp-	-ARG-	-Asn-	-Ser-	-Tyr-	-Pro-	-Gly-	-Cys-	-Pro-	-Ser-	EGF [42]
-Ala-	-Leu-	-Gly-	-Ser-	-Gln-	-Arg-	-Glu-	-Gly-	-ARG-	-Asn-	-Pro-	-Gln-	-Leu-	-Asn-	-Gln-	-Gln		GRP [45]
-Arg-	-Gly-	-Ala-	-Arg-	-Ala-	-Arg-	-Leu-	-Gly-	-ARG-	-Gln-	-Val-	-Asp-	-Ser-	-Met-	-Trp-	-Ala-	-Glu-	HGRH[39]
-Ala-	-Arg-	-Leu-	-Gly-	-Ala-	-Leu-	-Leu-	-Ala-	-ARG-	-Tyr-	-Ile-	-Gln-	-Gln-	-Val-	-Arg-	-Lys-	-Ala-	CC39[37]
-Tyr-	-Gly-	-Gly-	-Phe-	-Leu-	-Arg-	-Arg-	-Ile-	-ARG-	-Pro-	-Lys-	-Leu-	-Lys-	-Trp-	-Asp-	-Asn-	-Gln-	Dyn8[31]
-Gly-	-Gly-	-Phe-	-Met-	-Arg-	-Arg-	-Val-	-Gly-	-ARG-	-Pro-	-Glu-	-Trp-	-Trp-	-Met-	-Asp-	-Tyr-	-Gln-	Enk [46]
-Leu-	-Arg-	-Ala-	-Leu-	-Leu-	-Ala-	-Gly-	-Pro-	-ARG-	-Ser-	-Leu-	-Arg-	-Arg-	-Ser-	-Ser-	-Cys-	-Phe-	ANF [47]
-Gly-	-Ser-	-Pro-	-His-	-Ala-	-Ala-	-Val-	-Pro-	-ARG-	-Glu-	-Leu-	-Ser-	-Pro-	-Leu-	-Asp-	-Leu		PIP [10]

This is a partial list of sequences including only an example from a representative species and a single copy of a peptide when present in multiple copies, as in the case of FMRF-amide. The monobasic amino acid at the cleavage site is designated as 0, the positions C-terminus to the cleavage site are designated as +1, +2, etc and the positions N-terminus to the cleavage site are designated as -1, -2, etc. Abbreviations: Ref; References, Dyn B; Dynorphin B, SS28; Somatostatin 28, IGF1; Insulin-like Growth Factor 1, BNP; Brain Natriuretic Peptide, FMRF; FMRF-amide, Ant; Antrin, PanP; Pancreatic Polypeptide, CC58; Cholecystokinin-58, ELH; Egg-Laying Hormone, VNII; Vasopressin/Neurophysin II, HGRH; Human Growth Hormone Releasing Hormone, SS1; anglerfish somatostatin 1, β-En; β-endorphin, Caer; caerulein, Mega; megainin, Levi; levitide, RGRH; rat growth hormone releasing hormone, EGF; Epidermal Growth Factor, PHM; Peptide Histidyl Methionine, IGII; insulin-like growth factor II, CC8; cholecystokinin-8, GRP; Gastrin Releasing Peptide, CCK-39; cholecystokinin-39, Dyn8; dynorphin A-8, Enk; enkephalin, ANF; Atrial Natriuretic Factor, PIP; Pancreatic Icosa Peptide.

Arg in the dogfish β-End fits the rules and tendencies but the human β-End does not. The fact that only the dogfish β-End, and not the human β-End is processed further supports the view that the rules correctly predict a processing site. Variants of proinsulin [12] and pro-albumin [13] have been found in which the peptide is

Table II

Frequency of the residues in and around the 110 sequences containing monobasic cleavage site for a few select amino acids (presented as a percentage). The designation of sites is described in legend to Table I

	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4
Arg	12	15	10	4	76	4	0	81	0	2	4	8
Lys	16	3	1	4	6	0	0	19	0	1	8	6
Ser	6	8	3	4	0	6	0	0	36	9	7	22
Ala	7	8	0	7	2	0	10	0	12	11	12	7
Gly	4	5	3	0	0	2	40	0	21	0	14	9
Leu	4	10	1	11	2	14	4	0	0	14	2	5
Val	1	8	4	2	3	5	23	0	0	8	1	0

not processed and circulates in sera as the unprocessed precursor. Upon analysis of these variants, the amino acid sequence showed that one of the amino acids from a dibasic cleavage site was replaced by a His in the case of proinsulin and Gln in the case of proalbumin (Fig. 1). In these examples the resulting single basic amino acids do not fit the rules, and the lack of processing in vivo, lends further support to the validity of the proposed rules.

To see if these rules and tendencies could be extended to the dibasic cleavages, 70 dibasic sites that are known to be processed were examined. Approximately 70% of the examples fit all 4 rules and 97% fit at least 3 rules. In contrast, when the dibasic sites that are not cleaved were examined, only 16% fit all of the rules, and 45% fit at least three rules. Although these rules and tendencies are different from the observations described by Harris [14], there are some similarities. Harris noted that the primary recognition site involved in dibasic processing contains a monobasic or a strongly polar residue in the vicinity of the cleavage site.

The proposed rules and tendencies also could be extended to multibasic processing that occurs in the secretory pathway. Examples include: the formation of some peptide hormones, blood coagulation factors and receptors for insulin and insulin-like growth factor-I (IGF-I). A number of blood factors, such as factor VII, factor IX, factor X, prothrombin, and protein S, are synthesized as precursors that undergo post-translational processing at multibasic sites prior to secretion into the blood [15-20]. It has also been shown that the insulin receptor and IGF-I receptor require processing at a tetrabasic site to release the α and β subunits [21,22]. Carboxypeptidase E (CPE, an enzyme involved in peptide biosynthesis) is synthesized as a precursor that contains a pentabasic sequence and processing at the site would release mature CPE [23]. It is clear from comparing the sequences that the processing at multibasic sites also follows all the rules and some of the tendencies. The involvement of a basic amino acid at the -3 position is highlighted by the unprocessed naturally occurring variants of factor IX (Ox3 factor IX and factor $IX^{cambridge}$) and an insulin receptor mutant [15,24,25]. In Ox3 factor IX a mutation of the Arg at the -3 to Gln resulted in the absence of processing at the tetrabasic site [15]. In factor $IX^{cambridge}$ a mutation

	-7 -6 -5 -4 -3 -2 -1 0 +1 +2 +3 +4
Human PP	-Leu-Thr-Arg-Pro-Arg-Tyr-Gly-Lys-Arg-His-Lys-Glu-
Guinea pig PP	-Leu-Thr-Arg-Pro-Arg-Tyr-Gly-Lys-Ser-Ala-Glu-Glu-
Human β-End	-Phe-Met- -Thr- -Ser-Glu-Lys-Ser-Gln-Thr-Pro-
Dogfish β-End	-Phe-Met-Lys-Ser-Trp-Asp-Glu-Arg-Gly-Gln-Lys-Pro-
Proinsulin	-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-Arg-Gly-Ile-Val-
Proinsulin variant	-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-His-Gly-Ile-Val-
Proalbumin	Arg-Gly-Val-Phe-Arg-Arg-Glu-Ala-His-
Proalbumin variant	Arg-Gly-Val-Phe-Arg-Gln-Glu-Ala-His-

Fig. 1. Comparison of amino acid sequences around the cleavage sites. The designation of sites is in the legend to Table I. The basic amino acids involved in cleavages are underlined. PP = pancreatic polypeptide; β-End = β-endorphin.

of the cleavage site from Arg-Pro-Lys-Arg to Arg-Pro-Lys-Ser resulted in the absence of processing [24]. In the insulin receptor, a mutation of Arg-Lys-Arg-Arg to Arg-Lys-Arg-Ser resulted in the lack of processing [25]. The absence of a basic amino acid at the −3 position could explain the absence of processing in these two examples.

It could be hypothesized that one or several related endoproteases is/are involved in processing at monobasic, dibasic, and multibasic cleavage sites. A number of putative processing enzymes selective for dibasic [26] and monobasic [27] cleavage sites have been reported. In most cases, these enzymes have been reported to be specific either for dibasic or monobasic sites and it is therefore unlikely that a single enzyme performs both cleavages in vivo. Two monobasic peptide processing enzymes, a dynorphin converting enzyme from rat brain and a caerulein processing frog skin endoprotease, have been shown to have a requirement for a basic amino acid either at the −3, −5 or the −7 sites [28–30]. Also, the frog skin endoprotease does not tolerate an aliphatic amino acid at the +1 site (rule no. 2) and partially tolerates an aromatic amino acid at the −1 site (rule no. 4) [30]. It is exciting that the monobasic processing enzymes display substrate specificity consistent with the proposed rules that were generated entirely by comparing sequences. As more monobasic cleavage sites become known, it is likely that a further refinement of the rules and tendencies described here will be possible. A consensus site for predicting cleavage sites is important, both for the studies on peptides and processing enzymes. It is hoped that the present description of the rules for monobasic cleavage sites will be useful for these purposes.

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