Intracellular Proteolytic Processing of the Heavy Chain of Rat Pre-α-inhibitor

THE COOH-TERMINAL PROPEPTIDE IS REQUIRED FOR COUPLING TO BIKUNIN

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Pre-α-inhibitor is a serum protein consisting of two polypeptides named bikunin and heavy chain 3 (H3). Both polypeptides are synthesized in hepatocytes and while passing through the Golgi complex, bikunin, which carries a chondroitin sulfate chain, becomes covalently linked to the COOH-terminal amino acid residue of H3 via its polysaccharide. Immediately prior to this reaction, a COOH-terminal propeptide of 33 kDa is cleaved off from the heavy chain. Using COS-1 cells transfected with rat H3, we found that in the absence of bikunin, the cleaved propeptide remained bound to the heavy chain and that H3 lacking the propeptide sequence did not become linked to coexpressed bikunin. Sequencing of H3 secreted from COS-1 cells showed that part of the molecules had a 12-amino acid residue long NH2-terminal propeptide. Cleavage of this propeptide, which occurred in the endoplasmic reticulum, was found to require basic amino acid residues at P1, P2, and P3 suggesting that it is mediated by a Golgi enzyme in transit. Deletion of the NH2-terminal propeptide or blocking of its release affected neither transport nor coupling of the heavy chain to bikunin.

Bikunin is a 25-kDa serum protein whose polypeptide consists of two tandemly arranged proteinase inhibitor domains of the Kunitz type (1). Bikunin is made by hepatocytes and is synthesized as a precursor also containing α1-microglobulin (2, 3). Late during its intracellular transport, the bikunin precursor acquires a chondroitin sulfate chain and shortly afterward, α1-microglobulin is released by proteolytic cleavage (4). Just before this cleavage, part of the bikunin precursor molecules becomes covalently linked via the chondroitin sulfate chain to one or two polypeptides of about 80 kDa, named the heavy chains. In this fashion, pre-α-inhibitor (Pαl)1 is formed from one bikunin and one heavy chain and inter-α-inhibitor (Iαl) from one bikunin and two heavy chains. The polypeptide compositions of these proteins have been shown to differ between species. Iαl and Pαl from man and rat contain H1 and H2, and H3, respectively (5, 6); the corresponding bovine proteins contain H2 and H3, and H2, respectively (7). The physiological function of Pαl and Iαl is not clear but in vitro experiments have shown that the two proteins are required for the formation of the hyaluronan-containing matrix that surrounds certain cells (6, 8). Other results suggest that Iαl might have a role in inflammation (9).

Sequence analysis as well as pulse-chase experiments have revealed that the heavy chains have COOH-terminal propeptides of about 30 kDa (10–12). These extensions are apparently released by a proteolytic cleavage between an Asp and a Pro residue (13). The enzyme mediating this reaction is unknown and in the present study we have defined its specificity by mutating the amino acid residues near the cleavage site. It has been demonstrated that the chondroitin sulfate chain of bikunin is linked to the heavy chains through an ester bond between an internal GalNAc residue and the α-carbon of the COOH-terminal amino acid (5, 14). The mechanism for the formation of this bond is unknown but in this paper we present evidence that the COOH-terminal propeptide is required for the coupling reaction.

Comparison of the amino acid sequences near the site where signal peptides have been found to be cleaved has yielded a set of rules that can be used for predicting the cleavage site (15). When these rules are applied to the sequence deduced from the cDNA of rat H3, they indicate that the signal peptide ends 12 amino acid residues before the beginning of the mature polypeptide implying the existence of an NH2-terminal propeptide (6). Here we confirm this prediction experimentally, and show that the cleavage, although it occurs in the ER, is mediated by an enzyme whose sequence specificity is that of a Golgi proprotein convertase.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, fetal bovine serum, glutamine, and penicillin/streptomycin were obtained from Statens Veterinärmédecinska Anstalt (Uppsala, Sweden). Endo-β-N-acetylgalactosaminidase H (Endo H) and fish sperm DNA were from Boehringer-Mannheim. Brefeldin A, chondroitinase ABC, and antibodies against the c-Myc epitope were from Sigma. The expression vector pXM was from Genetics Institute Inc. (Cambridge, MA), pSecTag from Invitrogen (Leek, The Netherlands), oligonucleotides from DNA Technology (Aarhus, Denmark), restriction endonucleases from Amersham, and Pfu polymerase from Stratagene. Tran35S-label (>1000 Ci/mmol) was from ICN and 3H]leucine (52 Ci/mmol) and 3H]valine (44 Ci/mmol) from Amersham. cDNA for rat α1-microglobulin-bikunin was a gift from B. Åkerström (Lund University, Sweden).

Production of Specific Antiserum—An antisera against the putative NH2-terminal extension of rat H3, PBRSLRLIGKRC, was obtained commercially (Medprobe, Oslo, Norway); the corresponding peptide was conjugated to keyhole limpet hemocyanin with N-maleimidobenzyloxycarbonyl-N-hydroxysuccinimide ester and used for the immunization of a rabbit. An antisera against rat Pαl was obtained by injecting the protein intramuscularly into a rabbit; Pαl was purified from rat plasma as described previously (6). Antibodies specific for H3 were obtained from this anti-
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Results

Secretion of H3—The time course for the secretion of H3 from transfected COS-1 cells was determined by pulse-chase experiments followed by immunoprecipitation and SDS-PAGE (Fig. 1A). In pulse-labeled cells, the precursor of H3 (with an apparent molecular mass of about 113 kDa) was detected as a double band (indicated with H3). After 20 min of chase, the cleaved form of H3 appeared in the cells (indicated with H3); upon longer times of chase, it constituted maximally 10–20% of the total amount in the cell samples. Both uncleaved and cleaved H3 appeared in the medium after a lag period of 20–40 min and attained maximal levels after 40–80 min. The relative amount of cleaved H3 in the medium was 45–50%. When a sample from pulse-labeled cells was pretreated with endoglycosidase H (Endo H), an enzyme removing early forms of N-linked oligosaccharides (17), the upper band of pH3 disappeared showing that the heterogeneity was due to incomplete glycosylation (Fig. 1B, lane 2). The same treatment of a sample from cells chased for 40 min showed that the cleaved form of H3 was resistant to Endo H (lane 4) indicating that it had reached the medial Golgi complex. As expected, both cleaved and uncleaved H3 in the medium were resistant to the enzyme (lane 6).

The observations that the intracellular cleaved form of H3 appeared only after a distinct lag period and that it was Endo H-resistant indicated that the cleavage occurred late in the Golgi complex. To further test this notion, we exploited the effects of the fungal metabolite brefeldin A on protein secretion. In cells treated with this compound, protein transport ceases and enzymes normally residing in the cis, medial, or trans Golgi appear in the ER, where they will modify the retained secretory proteins (18). However, enzymes residing in the trans Golgi network or secretory vesicles will not be relocated in the presence of the drug, and the secretory proteins retained in the ER will therefore not be modified by these enzymes. As expected, treatment of the transfected COS-1 cells with brefeldin A prevented secretion of newly synthesized H3 (Fig. 1B, cf. lanes 8 and 10). Furthermore, the retained protein remained uncleaved (cf. lanes 7 and 9). These results suggest that the cleavage of the COOH-terminal propeptide normally takes place beyond the trans Golgi.

The COOH-terminal Propeptide Remains Associated with H3 after Cleavage—In the experiments described in Fig. 1A, a
cells expressing H3 precursor were labeled with \([35S]\)Met and the heavy chain was isolated from the medium by immunoprecipitation followed by SDS-PAGE and fluorography. The precipitated 33-kDa polypeptide, indicated with C in Fig. 4A, was excised from the gel and subjected to Edman degradation. The radioactivity recovered in each cycle is shown as bars. The amino acid sequence of the COOH-terminal propeptide is shown above the bars. Because of spillover in the sequential extractions, the radioactivity of a negative cycle following a positive one is elevated.

The COOH-terminal propeptide and H3 could be either that the propeptide was attached to the heavy chain or that the antiserum contained antibodies against the propeptide. The latter alternative does not seem to be the case since we found that the propeptide expressed alone in COS-1 cells was not recognized by the antiserum against the propeptide. The latter alternative does not seem to be the case since we found that the propeptide was not actually attached to H3, as determined by immunoprecipitation and detected by SDS-PAGE followed by fluorography. A and B show the result obtained with cells expressing H3 and the COOH-terminal propeptide, respectively. The precursor of H3, cleaved H3, and the COOH-terminal propeptide are indicated by pH3, H3, and C, respectively. Note that the propeptide is in the same fraction as H3 in A but has sedimented more slowly in B.

The reason for the coprecipitation of the COOH-terminal propeptide and H3 could be either that the propeptide was attached to the heavy chain or that the antiserum contained antibodies against the propeptide. The latter alternative does not seem to be the case since we found that the propeptide expressed alone in COS-1 cells was not recognized by the antiserum (result not shown). To test whether the COOH-terminal propeptide was actually attached to H3, we ascertained the sedimentation behavior of the propeptide in the presence or absence of the heavy chain. To this end, COS-1 cells expressing either the H3 precursor or only the propeptide (with a Myc tag) were labeled with \([35S]\)Met. Samples of the media were then analyzed by velocity centrifugation followed by immunoprecipitation and SDS-PAGE. This experiment showed that the COOH-terminal propeptide formed from the H3 precursor by intracellular proteolytic cleavage sedimented at the same rate as cleaved and uncleaved H3 (Fig. 3, upper panel) whereas the COOH-terminal propeptide expressed alone sedimented more slowly (lower panel). The simplest interpretation of these results is that the propeptide remains attached to H3 after cleavage.

The COOH-terminal Propeptide Is Needed for Coupling of H3 to Bikunin—The observation that there is an association between the COOH-terminal propeptide and the heavy chain suggested to us that the propeptide might have a role in the coupling of H3 to bikunin. To test this idea, we exploited our previous finding that coexpression of H3 and the bikunin precursor in COS-1 cells leads to coupling of the two polypeptides (6). The result of such an experiment is shown in Fig. 4 in which the cell medium of the transfected cells was analyzed with antibodies against bikunin (lane 1) or H3 (lane 2). The resulting protein complex, which has an apparent molecular mass of 180 kDa upon SDS-PAGE, is indicated with an arrow. To ascertain whether the COOH-terminal propeptide of H3 is necessary for the coupling reaction, we coexpressed bikunin and H3 lacking the propeptide; secretion of the truncated heavy chain was normal but no 180-kDa complex was formed (lane 3).
The analysis of the medium from cells coexpressing bikunin and H3 with antibodies against bikunin showed, as earlier reported (6, 19), that both bikunin precursor with and without chondroitin sulfate as well as mature bikunin were secreted; the respective protein bands are indicated with 1, 2, and 3 (Fig. 4, lane 1). When antibodies against H3 were used, there was a weak band at the position of the COOH-terminal propeptide (lane 2, indicated with C). The relative amount of this band was 2–3 times lower than that of the band appearing when H3 was expressed alone (Fig. 1A, lane 9), consistent with our conclusion that the propeptide is released upon coupling; the occurrence of the propeptide in this sample can be accounted for by presence of cleaved but uncomplexed heavy chain (indicated with H3). Furthermore, when the immunoprecipitation was done with antibodies against bikunin, there was even less radioactive material at the position of the propeptide (lane 1) and pretreatment with chondroitinase ABC reduced this amount to less than 1% of the radioactivity in the 180-kDa band (not shown).

Specificity of Cleavage at COOH Terminus—The amino acid sequence close to the cleavage site of the COOH-terminal propeptide of H3 is shown in Fig. 5A. Amino acid residues conserved in this protein in man, mouse, and rat (13) are in bold. As a first step in the identification of the enzyme cleaving the COOH-terminal propeptide, we determined which of the conserved residues are essential for cleavage. To this end they were mutated as shown in Fig. 5B. The corresponding polypeptides were then expressed in COS-1 cells and the degree of cleavage assessed by SDS-PAGE (Fig. 5C). This analysis showed that Asp at P3 and P1 as well as Pro at P9′ are absolutely required (<1% cleaved). Substitution of Glu for His at P9′ and Tyr for Phe at P9′ reduced cleavage approximately 3-fold whereas substitution of Val-Val for Ile-Ile at P9′ and P9′ was without effect. Interestingly, mutation of Val to Ala at P4 resulted in a significantly higher cleavage (lane 2).

Detection of NH2-terminal Propeptide—As mentioned in the Introduction, analysis of the cDNA of H3 suggests that the signal peptidase cleaves 12 amino acid residues before the beginning of the mature polypeptide implying the existence of an NH2-terminal propeptide. To experimentally test this hypothesis, we wanted to block the release of the putative propeptide and then determine the NH2-terminal sequence of the secreted proprotein. With the assumption that the two basic amino acid residues preceding the NH2 terminus of mature H3 (Fig. 6A, right-hand arrow) are essential for cleavage, we mutated these to Asn and Ser. The modified protein was then expressed in COS-1 cells which were labeled with [3H]Leu. The protein was isolated from the cell medium by immunoprecipitation followed by SDS-PAGE, subjected to Edman degradation, and the radioactivity released in each cycle was measured (Fig. 6B, closed bars). Elevated values were obtained at cycles 6, 8, and 9. This result is consistent with the cleavage of the signal peptide occurring between amino acid residues 21 and 22 (left-hand arrow in A); the corresponding sequence is shown above the bars. When the same experiment was done with cells labeled with [3H]Val (open bars), no significant increase of radioactivity was obtained, consistent with the absence of Val in the NH2 terminus of the propeptide.

As a control, wt H3 produced from COS-1 cells was also analyzed by radiosequencing. Labeling with [3H]Leu yielded elevated radioactivity values in cycles 2, 6, 8, and 9 (Fig. 6C, open bars). This result can be explained by the occurrence of similar amounts of H3 molecules with and without the NH2-terminal propeptide; the corresponding sequences are shown above the bars in plain and bold letters, respectively. Consistent with this conclusion, wt H3 labeled with [3H]Val yielded increased values at cycles 6 and 7 (open bars). A quantitative analysis of the data in Fig. 6C indicates that 35–40% of H3 was cleaved (average of two experiments).

Specificity of Cleavage at NH2 Terminus—Comparison of the amino acid sequences of H3 from man, mouse (13), and rat (6) shows that the basic amino acid residues near the cleavage site of the NH2-terminal propeptide (see Fig. 6A), at P5, P7, and P8, are conserved. As described above, simultaneous substitution of those at P9 and P7 against non-basic ones abolishes cleavage (Fig. 6B). To assess the relative importance of each of the conserved basic residues, we mutated them one at a time (Fig. 7A) and determined the degree of cleavage. For these experiments the immunoprecipitation was first done with antibodies against the NH2-terminal propeptide and subsequently with antibodies against the whole protein (denoted I and II, respectively); the amount of H3 obtained in the second extraction (relative to the amount obtained with both antibodies was taken as a measure of the degree of cleavage. These experiments showed that Asp at P3 and P1 as well as Pro at P9′ are absolutely required (<1% cleaved). Substitution of Glu for His at P9′ and Tyr for Phe at P9′ reduced cleavage approximately 3-fold whereas substitution of Val-Val for Ile-Ile at P9′ and P9′ was without effect. Interestingly, mutation of Val to Ala at P4 resulted in a significantly higher cleavage (lane 2).

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showed that 33–37% of wt H3 was cleaved (lanes 1 and 2), which agrees with the result obtained by amino acid sequencing (Fig. 6C). The proportion of H3 lacking the COOH-terminal extension was the same in the samples obtained with the two different antibodies, implying that the NH2-terminal propeptide does not affect the subsequent cleavage of the COOH-terminal propeptide. Substitution of one of the basic amino acids, reduced the cleavage to 7–15% (Fig. 7B, lanes 3–8). With the same type of analysis in which the residues at P1 and P2 had both been mutated, no cleavage was detected (<1%). When this mutant was coexpressed with bikunin, the same amount of the 180 kDa complex was detected in the medium as with the wild type protein (not shown). Analysis of wt H3 from cells labeled for 5 min yielded essentially the same degree of cleavage as for the secreted form (not shown) implying that processing of the NH2-terminal propeptide occurred in the ER.

**DISCUSSION**

Many secretory proteins are synthesized as precursors that are proteolytically cleaved during their transport to the cell surface. Normally this cleavage occurs just before the proteins are released: in the trans Golgi network or in the secretory vesicles (20). In several cases the cleavage has been shown to make the proteins biologically active suggesting that an early activation would be harmful to the cell (21). The cleavage of proproteins typically occurs next to two basic residues and recently, a family of enzymes was identified that mediates this reaction (22).

In this study we have used the heavy chain of rat Pol, heavy chain 3 (H3), as a model for the proteolytic processing of the heavy chains of the bikunin-containing proteins. The cleavage sites for the COOH-terminal propeptides of these proteins differ from those of other proproteins in that they lack adjacent basic amino acid residues (23). Using site-directed mutagenesis we have now shown that five of the conserved residues flanking this site in H3 are essential for cleavage; to our knowledge there is no known proteinase that recognizes the corresponding sequence. We also found that the COOH-terminal propeptide (in the absence of bikunin) remains attached to the heavy chain. The conserved amino acid residues near the cleavage site that we found not to be required for cleavage, P6, P7′, and P8′, might mediate this interaction. The degree to which the heavy chains are cleaved during secretion varies greatly between different cell types. Thus, experiments with human hepatocytes have shown that H3 and H2 are completely cleaved in these cells whereas in the human hepatoma cell line Hep G2, they are partially or not processed at all, respectively (12); these observations indicate that the cleavage is not autocatalytic.

We have earlier shown that coexpression of bikunin and heavy chain in COS-1 cells leads to coupling of the two polypep-
For many proteins, the propeptides have been found to be required for proper folding (25). This does not appear to be the case for the NH$_2$-terminal propeptide of H3, as judged by the fact that when H3 was expressed without the NH$_2$-terminal propeptide, it was secreted normally and could be coupled to bikunin. However, absence of the NH$_2$-terminal propeptide during synthesis might affect other properties of the protein adversely, perhaps those associated with its function in the formation of the hyaluronan-containing matrix that surrounds various cell types (26–28).

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