Intracellular Coupling of the Heavy Chain of Pre-α-inhibitor to Chondroitin Sulfate*

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Pre-α-inhibitor is a serum protein consisting of two polypeptides, the heavy chain and bikunin, covalently linked through an ester bond between the chondroitin sulfate chain of bikunin and the α-carboxyl group of the carboxyl-terminal residue of the heavy chain. The heavy chain is synthesized with a carboxyl-terminal extension, which is cleaved off just before the link to bikunin is formed. Our earlier studies indicate that this extension mediates the cleavage, and we have now found that a short segment on the amino-terminal side of the cleavage site is also required for the reaction. Furthermore, we previously showed that coexpression of the heavy chain precursor and bikunin in COS-1 cells leads to linkage, and we have now used this system to identify a His residue in the carboxyl-terminal extension that is specifically required for the intracellular coupling of the two proteins. In addition, we have shown that another chondroitin sulfate-containing protein, decorin, will also form a complex with the heavy chain, as will free chondroitin sulfate chains. These results suggest that in vivo there might be other, as yet unknown, chondroitin sulfate-containing polypeptides linked to the heavy chain.

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Bikunin (Bk) is a 25-kDa chondroitin sulfate-containing protein consisting of two Kunitz-type protease inhibitor domains, which is secreted by hepatocytes (reviewed in Ref. 1). The physiological function of bikunin is unknown, but recent in vitro studies suggest that it might play a role in inflammatory reactions (2, 3). In blood plasma, most Bk occurs in complex with one or two polypeptides of 75–80 kDa named the heavy chains, which are homologous to each other. The corresponding proteins are pre-α-inhibitor (PoI) bikunin linked with heavy chain 3 and inter-α-inhibitor bikunin linked with heavy chains 1 and 2 (4). These proteins have been shown to be required for the stabilization of the cumulus cell-oocyte complex (5), as well as for the formation of the hyaluronan-containing coat on fibroblasts and mesothelial cells (6). In inflamed tissues (2) and in cell cultures of fibroblasts (7), the heavy chains have been found to be transferred to hyaluronan molecules. The physiological significance of this process is not known, but in vitro experiments have shown that hyaluronan molecules bearing heavy chains are less sensitive to degradation by free radicals (8).

Bikunin is synthesized in a precursor form that has α-carboxyl microglobulin at its amino terminus (9, 10), and the two proteins are released through a proteolytic cleavage late in the Golgi complex (11, 12). The heavy chains are synthesized with a carboxyl-terminal extension (CTX), which is cleaved off when the proteins reach the Golgi complex (13, 14). Both bikunin and the heavy chains are synthesized in hepatocytes, and immediately after the cleavage of the CTX, they become linked through an ester bond between the chondroitin sulfate chain of bikunin and the α-carboxyl group of the carboxyl-terminal amino acid residue of the heavy chains (4, 15) (Fig. 1). The molecular mechanism of this assembly process is still poorly known. We have previously shown, however, that coexpression of the precursors of bikunin and the heavy chain of PoI (heavy chain 3: H3) in COS-1 cells leads to coupling (16), and we have used this system to investigate both the cleavage and coupling reactions. These studies provided evidence that the CTX cleavage occurs through an intramolecular reaction (17, 18).

In the present study we have investigated whether the part of the heavy chain precursor corresponding to mature H3 has a role in CTX cleavage. Our results show that an 8-amino acid residue segment preceding the cleavage site is necessary and sufficient for this reaction. We have also identified a His residue (His46) in the CTX that is essential for the coupling of H3 to the bikunin precursor (but not for CTX cleavage). In addition, we have found that the α-carboxyl microglobulin part of the bikunin precursor is not essential for the coupling and that H3 can be linked to the chondroitin sulfate of a proteoglycan other than bikunin, as well as to free chondroitin sulfate chains. Because bikunin is the most abundant CS-containing proteoglycan produced by hepatocytes and so far the only protein known to be covalently linked to the heavy chains, our results suggest that there might be other, minor chondroitin sulfate-containing proteins linked to heavy chains in vivo.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, fetal bovine serum, and glutamine were obtained from Stata-new Veterinary Medicinals, Anstalt (Uppsala, Sweden) and pXCM and pScTag plasmids from The Genetics Institute, Inc. (Cambrigde, MA) and Invitrogen (Leek, The Netherlands), respectively. Oligonucleotides were from DNA Technology (Aarhus, Denmark), Tran5S label (>1000 Ci/mmole) and [35S]sulfate from ICN (Costa Mesa, CA), and chondroitinase ABC, p-nitrophenyl-β-D-xolphynoroside (pNPX), and protease inhibitor mixture (C3667) were from Sigma. PoI was purified from rat plasma as previously described (16), and antisera was produced in a rabbit. Antiserum against rat serum albumin was a gift from Dr. Å. Oldberg, Lund University, Sweden.

Construction of Expression Vectors—cDNA for mouse albumin was obtained by PCR from mouse liver cDNA (Marathon-Ready, CLONTECH). The primers used were: 5′-ACTACGTGACATGAAGTGGG-
respectively. The fusion constructs described above, as well as the expression vector pXM. The bikunin coding sequence was amplified by PCR at the 3' ends (Val-Asp) at the linkage region of each construct was introduced by PCR at the 3' and 5' ends of the albumin and pH3 fragments, respectively. The fusion constructs described above, as well as the cDNAs coding for rat pH3 (16) and human decorin (a gift from Dr. A. Oldberg, Lund University, Sweden), were cloned into the eukaryotic expression vector pCM. The bikunin coding sequence was amplified by PCR using rat α1-microglobulin-bikunin cDNA (a gift from B. Åkerström, Lund University, Sweden) and subcloned into the pSecTagB expression vector in-frame with an Ig κ-chain signal sequence. Substitutions of amino acid residues were made with the unique site elimination procedure (U.S.E. mutagenesis kit, Amersham Biosciences AB, Sweden). All constructions were verified by DNA sequencing.

Transfection and Metabolic Labeling—COS-1 cells were transfected by electroporation as described previously (16). Two days later, the cells were rinsed twice with phosphate-buffered saline and metabolically labeled in the presence of either [35S]methionine or [35S]SO4-2. For protein labeling, the cell cultures were incubated in 1.0 ml of Eagle’s minimal essential medium supplemented with 2 mM methionine, 2 mM glutamine, and [35S]methionine (0.1 mCi/ml) for 4 h at 37 °C. For labeling of sulfated polysaccharides, 1.0 ml of Eagle’s minimal essential medium lacking SO4-2 and supplemented with 2 mM glutamine, 1 mg/ml bovine serum albumin, and [35S]SO4-2 (0.1 mCi/ml) was used, and the cells were incubated for 4 h at 37 °C.

Immunoprecipitation—The medium of labeled cells was subjected to immunoprecipitation, and subsequent electrophoretic analysis was done as previously described (16), except that the radioactivity detection was done by phosphorimaging (Bio-Imaging Analyzer, Fujifilm).

Induction of Free Chondroitin Sulfate Synthesis—COS-1 cells transfected with pH3 were incubated at 37 °C with various concentrations of pNFX or with vehicle alone (3% MeSO4) in 1.0 ml of labeling medium. After 30 min, either [35S]methionine or [35S]SO4-2 (0.1 mCi) was added to the cultures, and the incubation was continued for 4 h.

Chondroitinase Treatment—The immunoprecipitates were resuspended in 30 μl of 50 mM Tris-HCl pH 8.0, supplemented with a protease inhibitor mixture (to the final concentration of 26 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 μM aprotinin, 0.53 mM leupeptin, 0.90 mM bestatin, 0.38 mM pepstatin A, and 0.35 mM E-64) and then incubated with or without 5 milliunits of chondroitinase ABC for 5 h at 37 °C. Subsequently, the samples were subjected to SDS-PAGE, and radioactivity was detected by phosphorimaging.

RESULTS

Structural Requirements for Cleavage of the Carboxyl-terminal Extension of H3—During the secretory transport of the pH3 in hepatocytes, the CTX is cleaved off (Fig. 1). This reaction also occurs, although at a lower efficiency (40–60%), when pH3 is expressed in COS-1 cells. Based on results obtained in this system after the introduction of various mutations, we earlier concluded that the CTX, as well as a few adjacent amino acid residues on the amino-terminal side of the cleavage site, play a crucial role in the cleavage process (17). In the present study we wanted to determine whether other regions of the precursor are also engaged in this reaction. Therefore, amino-terminal segments of different length were deleted, and the remaining polypeptides preceded by the native signal peptide were expressed in COS-1 cells. Analysis of the culture medium showed, however, that these truncated forms were poorly secreted, presumably because of misfolding (data not shown). To circumvent this problem, we instead fused the amino-terminal segment of the truncated pH3s to the carboxyl terminus of albumin (Fig. 2A, lanes 3, compare with lane 1, the arrow indicates the complex consisting of H3 and bikunin precursor). On the contrary, a His649 mutant (which displayed reduced CTX cleavage) did not show any detectable coupling (lane 2). Thus, His649 seems to be specifically required for the coupling reaction. Mutation of Gly789 abolishes cleavage of the CTX (17) and, as expected, coexpression of this mutant with the bikunin precursor did not result in the formation of any complex (lane 5).

Structural Requirement in Bikunin Precursor for Coupling—The reason why bikunin is synthesized as a precursor is not known. α1-microglobulin is released after coupling of the precursor to the heavy chain that is about 8 amino acid residues long.

His493 in CTX Is Essential for Coupling of H3 to Bikunin—We have earlier identified amino acid residues near the cleavage site of rat pH3 that are crucial for the cleavage process (17). In the present investigation, we wanted to determine whether the conserved amino acid residues in this region that were found to have little or no influence on cleavage are instead essential for the subsequent coupling to the bikunin precursor. We therefore coexpressed bikunin and mutated H3 precursors in COS-1 cells and then assessed coupling by electrophoretic analysis of the secreted proteins as earlier described (17). pH3 mutated at Phe651 or at His653-655 seemed to yield the same degree of complex formation as that obtained with the wild type protein (Fig. 3, lanes 3 and 4, compare with lane 1, the arrow indicates the complex consisting of H3 and bikunin precursor). On the contrary, a His649 mutant (which displayed reduced CTX cleavage) did not show any detectable coupling (lane 2).

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labeled with \([35S]\)methionine for 4 h. The culture media were immunoprecipitated with antibodies against albumin or \(P1\) containing wild type pH3 (lane 3, \(wt\)) or fusions of albumin with the pH3 (lanes 4, 5), respectively.

FIG. 2. A short segment on the amino-terminal side of the cleavage site is required for intracellular cleavage of the H3 precursor. A, the H3 precursor, consisting of mature H3 (in black) and the CTX (in white; compare Fig. 1), was truncated from its amino terminus at different sites, and the remaining carboxy-terminal fragments were fused to the carboxy terminus of albumin (hatched rectangle; \(V D\) shows the Val-Asp residues derived from a SaII restriction site and present in all constructs). The amino acid sequence around the cleavage site between H3 and the CTX (closed arrow head) is shown with the different fusion sites as open arrow heads and the amino acid residues conserved between four species in bold. The albumin-pH3 chimeraS were then expressed in COS-1 cells, and cleavage was assessed as described below. The results are shown below the respective fusion site: chimeras yielding more than 40% cleavage are indicated with (+), those with 5–15% cleavage or completely resistant with (−) or (−), respectively. B, COS-1 cells were transiently transfected with pXM containing wild type pH3 (lane 3, \(wt\)) or fusions of albumin with the pH3 fragments remaining after the deletion of 639 (Δ639, lane 1) or 643 (Δ643, lane 2) amino-terminal amino acid residues. The cells were then labeled with \([35S]\)methionine for 4 h. The culture media were immunoprecipitated with antibodies against albumin or \(P1\) as indicated below the lanes and analyzed by SDS-PAGE followed by phosphorimaging. The upper and lower bands correspond to the uncleaved protein and the protein lacking the CTX, respectively. Note the lack of cleavage in lane 2. The positions of standard proteins run in parallel are shown to the right with their molecular masses in kDa.

CHARIDE (\(Bk-CS\); the double band results from differences in N-linked glycosylation, see Ref. 11). Thus, it appears that bikunin is able to form a chondroitin sulfate-linked complex with H3 in the absence of the \(\alpha\)-microglobulin part of the precursor.

Coupling of H3 to Decorin—We then wanted to examine whether the polypeptide part of bikunin is needed for coupling to H3. For this purpose we used another chondroitin sulfate-containing protein, decorin, which is synthesized by most types of connective tissue and consists of a 45-kDa core polypeptide chain and a chondroitin/dermatan sulfate chain of variable size (19). Expression of decorin in COS-1 cells and immunoprecipitation of the secreted proteins with antiserum against decorin yielded a diffuse band of 50–90 kDa (Fig. 5, lane 2, \(Dec+CS/DS\)) and a more distinct band of 45 kDa (\(Dec-CS/DS\)). These bands most likely represent decorin with or without chondroitin/dermatan sulfate, respectively, because only the slowly migrating band was sensitive to treatment with chondroitinase ABC (lane 3). When decorin was coexpressed with pH3, a 150–200-kDa complex was detected in the cell culture medium by immunoprecipitation with either decorin or \(\alpha\)-inhibitor antiserum (lanes 4 and 5, respectively; \(\text{square bracket-asterisk}\)). Chondroitinase ABC treatment of the immune complexes obtained with decorin antiserum resulted in the disappearance of the complex and generated a band with the electrophoretic mobility of H3 (lane 5, arrow, compared with lane 8). When \(\alpha\)-inhibitor antibodies were used for the immunoprecipitation, subsequent chondroitinase ABC treatment resulted in the
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formation of a new band with the mobility of decorin lacking glycosaminoglycan (lane 7, arrow, compared with lane 3). Taken together, these results indicate that upon coexpression of pH3 and decorin, the two proteins form a complex linked by a chondroitin sulfate chain.

Coupling of H3 to Free Chondroitin Sulfate—The finding that the polypeptide part of bikunin is not essential for the formation of the H3-glycosaminoglycan linkage prompted us to ascertain whether H3 could be coupled to free chondroitin sulfate chains. It has previously been demonstrated that such polysaccharides are synthesized and secreted upon incubation of cells with certain xylosides, such as pNPXP (20). Thus, we labeled COS-1 cells expressing H3 precursor with [35S]methylamine in the presence of increasing concentrations of pNPXP. Analysis of the culture medium with pNPXP antiserum revealed the formation of complexes larger than mature H3 and of a broad range of sizes (Fig. 6A, square bracket, lanes denoted (–)). Furthermore, the amount of H3 decreased with increasing concentration of pNPXP. Treatment of the immunoprecipitates with chondroitinase ABC (lanes denoted (+)) resulted in the disappearance of the polydisperse molecules and a concurrent increase in the amount of free H3. To confirm the presence of chondroitin sulfate in the complexes formed, we incubated cells expressing H3 precursor with pNPXP and [35S]sulfate; under the conditions used, mainly sulfated glycosaminoglycans were labeled. Analysis of the culture medium with antiserum against pNPXP showed the formation of H3-containing complexes with apparent molecular masses higher than 200 kDa at a pNPXP concentration of 3.1 mM (Fig. 6B, lane 3). At higher xyloside concentrations, complexes of lower molecular masses (90–120 kDa) were more abundant. Treatment of the immune complexes with chondroitinase ABC led to the disappearance of the H3-containing high molecular mass material. Taken together, these results indicate that the H3-containing complexes secreted upon expression of pH3 in the presence of pNPXP were H3 molecules linked to free chondroitin sulfate chains of variable length.

DISCUSSION

The most important finding of this study has been that the intracellular coupling of H3 to chondroitin sulfate does not depend on the polypeptide bearing the polysaccharide. In fact, we found that H3 could be coupled to free chondroitin sulfate chains (Fig. 6). Although our results were obtained through transfection experiments, they raise the possibility that chondroitin sulfate-bearing proteins other than bikunin might become linked to H3 in vivo. H3 has so far been shown to be synthesized only by hepatocytes (21, 22). In these cells, bikunin is by far the most abundant chondroitin sulfate-containing protein (11, 23), which could explain why only this protein has been found to be linked to H3. Perhaps more sensitive detection techniques would reveal that hepatocytes secrete other heavy chain-linked proteins. As judged by in situ hybridization experiments, low levels of pH3 transcript are present in certain non-hepatic organs such as the brain (21). It remains to be seen whether the corresponding protein is indeed produced in these tissues and if so, in what form.

Based on results obtained with pH3 expressed in COS-1 cells, we previously concluded that the intracellular cleavage of the carboxyl-terminal extension of pH3 is triggered by the low pH in the trans Golgi and occurs through an autocatalytic intramolecular reaction (18). Furthermore, we found that various recombinant modifications of the CTX abolished cleavage, indicating that this part of the protein is essential for the reaction (17). Whether the remainder of the protein is also involved in the cleavage process was not investigated. To address this issue, we have in the present investigation expressed chimeric proteins consisting of albumin and carboxyl-terminal...
fragments of pH3 of various lengths (Fig. 2B). Albumin folds quickly into a globular structure (24) with a flexible carboxyl-terminal end (25), making it likely that in the chimeric proteins, albumin and the pH3 fragments formed separate domains with their functional properties preserved. Support for this assumption comes from the observation that cleavage of the CTX in these constructs was sensitive to a lowering of pH, similar to the wild type protein (results not shown). Analysis of the different chimeric proteins showed that cleavage, in addition to the CTX, required a short (about 8 amino acid residues long) segment on the amino-terminal side of the cleavage site (Fig. 2A). Interestingly, the corresponding residues (Fig. 2A, in bold) are conserved between the H3s of four different species (rat, mouse, hamster, and man), suggesting that this region of H3 may be involved in specific interactions or have a specific conformational necessity for efficient cleavage.

Expression of pH3 in different cell lines has shown that the capacity to cleave the protein varies between cells, possibly because of differences in the pH of their trans Golgi (18). Should it be established that certain non-hepatic cells do secrete free pH3 in vivo, it is tempting to speculate that this form of the protein may have a different function than that of PaI. The autocatalytic cleavage might then serve as an activation step occurring either intracellularly, as indicated by our experiments, or extracellularly, where inflammation could lower the pH (26).

It was recently reported that the blood of mice lacking bikunin contains only the proform of the heavy chains (5). Whether the absence of the cleaved form is due to lack of autocatalytic cleavage in hepatocytes or to rapid plasma clearance remains to be investigated. Although the pH-dependent cleavage of pH3 appears to be autocatalytic, it is possible that cleavage associated with coupling to chondroitin sulfate occurs by a non-autocatalytic process requiring the presence of the polysaccharide. Studies with heavy chain 1 and 2 support this view (14, 28).

We reported earlier that if the CTX is deleted from pH3, no coupling takes place upon coexpression with bikunin (17). Because this modification was relatively large and likely to cause a conformational change in the remaining polypeptide, the obtained result cannot simply be taken as evidence that the CTX mediates coupling. In the present study we found, however, that the mutation of a single amino acid residue in the CTX (His646Gln) completely abolished coupling (Fig. 3). These findings suggest a specific role of His646 in the coupling process. Because chondroitin sulfate and His residues will have opposite charges at the pH occurring in the Golgi complex, it is possible that His646 might contribute to the initial interaction with the glycosaminoglycan preceding its covalent linkage to the heavy chain. Further evidence that the CTX has a central role in the coupling reaction comes from the observation that coexpression of bikunin and a chimeric protein consisting of albumin, 8 carboxyl-terminal residues of the mature H3, and CTX led to coupling; thus, the major part of the mature H3 molecule does not seem to be involved in this process. The mechanism of the coupling reaction is still unknown. Analysis of the sequence of CTX has revealed some similarity with multicopper-oxidases, but the significance of this finding is unclear (27). Our attempts to achieve coupling by incubating secreted bikunin and pH3 have so far been unsuccessful, possibly because as yet unidentified auxiliary factors are required.

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