Intracellular coupling of bikunin and the heavy chain of rat pre-α-inhibitor in COS-1 cells

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Pre-α-inhibitor is a serum protein consisting of two polypeptides: bikunin of 16 kDa, which carries an 8 kDa chondroitin sulphate chain, and heavy chain 3 (H3) of 74 kDa. The two polypeptides are linked through an ester bond between an internal *N*-acetylgalactosamine residue of the chondroitin sulphate chain and the C-terminal aspartic acid residue of H3. Both bikunin and H3 are synthesized by hepatocytes and become linked as they pass through the Golgi complex. H3 is synthesized with both N- and C-terminal extensions which are released during intracellular transport. To be able to analyse the assembly of pre-α-inhibitor in detail, we have cloned and sequenced the cDNA of rat H3. Upon expression of the protein in COS-1 cells, both propeptides were found to be released. Furthermore, co-expression of H3 and bikunin resulted in the two polypeptides becoming coupled, indicating that cells other than hepatocytes may have the capacity to form chondroitin sulphate-containing links.

INTRODUCTION

Bikunin is a chondroitin sulphate-containing serum protein whose polypeptide consists of two tandemly arranged proteinase inhibitor domains of the Kunitz type [1]; it was recently given the name bikunin and is also known as urinary trypsin inhibitor and acid-stable trypsin inhibitor [2]. In blood plasma, most bikunin occurs in complex with one or two similar polypeptides of 70–80 kDa named the heavy chains; three different heavy chains have been identified to date: H1, H2 and H3 [3]. Bikunin is linked to the heavy chains through an ester bond between its chondroitin sulphate chain and the α-carbon of the C-terminal amino acid of the heavy chains [4,5]. In human plasma, the two major bikunin-containing proteins are inter-α-inhibitor (IαI) and pre-α-inhibitor (PαI), the former protein being 3–4-fold more abundant than PαI [6]. The chain compositions of the bikunin-containing proteins have been shown to differ between species; human IαI and PαI contain H1 and H2, and H3, respectively [4], whereas the corresponding bovine proteins contain H2 and H3, and H2, respectively [7]. In the present study we have characterized rat IαI and PαI with respect to their chain compositions and plasma concentrations.

The physiological function of bikunin in its free form is not clear, but the protein has been shown to act as a growth regulator for endothelial cells and fibroblasts [8,9] and to inhibit the metastasis of cancer cells [10]. PαI and IαI have been found to stabilize the hyaluronan-containing coat on cumulus–oocyte complexes [11,12] as well as that on normal human fibroblasts and mesothelial cells [13]. The heavy chains in these structures seem to be covalently linked to hyaluronan through their C-terminal amino acid residue [14]. Thus it appears that the heavy chains have been released from the chondroitin sulphate chain through a substitution reaction [15]. These observations suggest that the role of bikunin in PαI and IαI is to keep the heavy chains in a state in which they can become linked to hyaluronan.

Bikunin is synthesized in the liver as a precursor which also contains α_1-microglobulin [16,17], a 28 kDa protein with immunoregulatory properties [18]. As the precursor passes through the Golgi complex, it acquires the chondroitin sulphate chain, and shortly thereafter bikunin and α_1-microglobulin are released by proteolytic cleavage at a dibasic sequence [19,20]. The heavy chains are synthesized in the same cells as the bikunin precursor and become linked to this polypeptide after it has acquired the chondroitin sulphate chain [19]. The heavy chains are synthesized with both N- and C-terminal extensions which are cleaved off during intracellular transport [21–23]. Whether these parts of the polypeptide play a role in the assembly of the bikunin-containing proteins is not known. Clearly, the availability of a system in which bikunin and the heavy chains could be expressed from the respective cDNAs would greatly facilitate further analysis of the assembly process. In a recent study it was shown that the hepatoma cell line HepG2 could not properly assemble bikunin-containing proteins, a finding suggesting that only primary hepatocytes have this capacity [23,24]. In the present paper we show, however, that cotransfection of COS-1 cells with cDNAs for rat H3 and bikunin leads to the coupling of the two polypeptides.

MATERIALS AND METHODS

Chemicals

Restriction endonucleases, T4 DNA ligase, Klenow polymerase, Sequenase 7-deaza-dGTP Sequencing kit, Megaprime labelling kit, Taq polymerase, [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{35}S]-dATP (1000 Ci/mmol) were obtained from Amersham (Bucks., U.K). PCR primers were from Scandinavian Gene Synthesis AB (Köping, Sweden) and hexadeoxy nucleotides from Pharmacia (Uppsala, Sweden). pGEM-2, pGEX and pXM were from Promega (Madison, MI, U.S.A.), Pharmacia (Uppsala, Sweden) and Genetics Institute Inc. (Cambridge, MA, U.S.A.) respectively. pSVF3 and cDNA for α_1-microglobulin–bikunin were gifts from M. Sjöberg (Karolinska Institute, Stockholm, Sweden) and B. Åkerström (Lund University, Sweden) respectively. A 2 kb fragment of the cDNA coding for human H3 was a gift from J.-P. Salier (INSERM U78, Boisguillome, France). Antiserum against rat serum proteins was obtained from Dakopatts (Glostrup, Denmark). Dulbecco's modified Eagle's medium (DMEM),

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EndoH, endo-β-*N*-acetylglucosaminidase H; H1, 2 and 3, heavy chains 1, 2 and 3 respectively; IαI, inter-α-inhibitor; PαI, pre-α-inhibitor.

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fetal bovine serum, glutamine and penicillin/streptomycin were obtained from Statens Veterinärmedicinska Anstalt (Uppsala, Sweden). Glasgow medium and tryptose phosphate broth were purchased from Gibco-BRL, Life Technology.

Isolation and characterization of cDNA clones

A cDNA library from a normal rat liver, constructed in the unique *Eco*RI site of bacteriophage γgt11, was purchased from Clontech (Palo Alto, CA, U.S.A.). The library was screened by plaque hybridization with a probe for the cDNA of H3. pGEM-2 vector was used for subcloning of the fragments obtained from the library screening. The DNA clone inserts were characterized by partial restriction-site mapping and 200 bp–600 bp fragments were subcloned into pGEM-2 vector. The inserts were then sequenced by the dideoxy chain-termination method on both strands at least twice. A clone containing the most 5′-terminal cDNA of H3 was obtained from a PCR in which 5′-RACE-(rapid amplification of cDNA ends) ready cDNA (Clontech) was used as a template. Two antisense primers were used: 5′-CGCAACTTCCTTCTCCTTGACACTCCC-3′ for the primary and 5′-TGCAAGCTTGAAACTTCCTTGGCCTTGTCTGC-ACG-3′ for the nested reaction. The amplified product was cleaved with *Eco*RI and *Hin*dIII and cloned into the pGEM-2 vector. The sequences were aligned and decoded with the aid of a computer program (Lasergene; DNASTAR Inc., Madison, MI, U.S.A.). Finally, a full-length cDNA was prepared by ligation of the appropriate fragments.

Preparation of antibodies

Fusion proteins were obtained with the vector pGEX-5T, which contains a sequence coding for a C-terminal histidine-hexa-peptide followed by glutathione S-transferase [25]. cDNA coding for a peptide with the sequence KGHGALNDLTFTEE-VDMKEM, which represents the amino acid residues 587–621 of rat H3, was obtained by PCR and inserted into the vector. DNA coding for bikunin was also inserted into the pGEX-5T vector. The proteins were expressed in *Escherichia coli*, purified in a single step by affinity chromatography on immobilized glutathione (Pharmacia, Uppsala, Sweden) and injected intra-muscularly into rabbits. Upon analysis of serum by immuno-blotting, both antisera recognized the expected polypeptides. The antiserum against bikunin reacted with the antigen in native form, whereas the antiserum against H3 did not. An antiserum against PαI and IαI was obtained by injecting a mixture of the isolated proteins into a rabbit. For the preparation of an antiserum specific for the heavy chains, bikunin was coupled to gel beads. Then a fraction of the antiserum against PαI and IαI was mixed with the beads, which were removed after 30 min by centrifugation. The bikunin was purified from rat urine as described [26] and coupled to CNBr-Sepharose (Pharmacia; Stockholm, Sweden) according to the manufacturer's instructions. For the sake of simplicity, these antibodies will be referred to as anti-PαI and anti-H3.

Purification of IαI and PαI from rat serum

Chelating Sepharose Fast Flow (20 ml; Pharmacia, Uppsala, Sweden) was activated with Zn^{2+}, as recommended by the manufacturer, and equilibrated with PBS containing 0.8 M NaCl. Rat serum (15 ml) was diluted five times with 4 M NaCl and filtered (0.45 μm). The serum sample was then applied at a linear flow rate of 10 cm/h and the gel was washed with 200 ml of PBS/0.8 M NaCl. Bound proteins were eluted with 400 ml of a gradient from 0 to 25 mM histidine in the same buffer. The presence of IαI and PαI in the collected fractions was assayed by SDS/PAGE, followed by blotting and detection with antibodies against bikunin. Fractions containing IαI and PαI were pooled and dialysed against 50 mM histidine/HCl/0.1 M NaCl, pH 5.5. The sample was applied on an anion-exchange gel (20 ml of Q-Sepharose HP; Pharmacia), equilibrated with the same buffer at a linear flow rate of 10 cm/h. The proteins were eluted with a 400 ml gradient from 0.1 to 0.5 M NaCl in 50 mM histidine/HCl, pH 5.5. The fractions (5 ml) were analysed by SDS/PAGE followed by immunoblotting. Those containing IαI and PαI (the tail of the major protein peak) were pooled and dialysed against 25 mM Tris/HCl, pH 8.0. This sample was applied on a heparin–Sepharose gel (30 ml; Pharmacia) at a linear flow rate of 3 cm/h. The flow-through was collected and the gel was washed with 300 ml of 25 mM Tris/HCl, pH 8.0/0.1 M NaCl. Finally, the bound proteins were eluted with 25 mM Tris/HCl, pH 8.0, containing 0.25 M NaCl. The fractions were analysed as described above and those containing pure IαI, the tail of the major protein peak, were pooled and the volume reduced to 1 ml by ultrafiltration. The fractions containing PαI, the flow-through of the heparin column, were pooled and dialysed against PBS. Further purification was performed with dye-affinity chromatography: the sample was applied at a linear flow rate of 20 cm/h on a column with 10 ml of Reactive Green 5 gel (Sigma, St. Louis, MO, U.S.A.). The material that passed through contained pure PαI.

For amino acid sequencing, the purified proteins were treated with 50 mM NaOH for 10 min, as described previously [27], and subjected to SDS/PAGE. The separated polypeptides were transferred to a PVDF membrane and stained with Coomassie Brilliant Blue. The relevant bands were excised and subjected to automated Edman degradation.

Construction of expression vectors and mutagenesis

The cDNAs for rat H3 and rat α_1-microglobulin–bikunin were subcloned into the eukaryotic expression vector pXM. This vector provides adenovirus late promoter-driven expression of introduced cDNA. cDNA of rat H3 was also subcloned into the pSVF3 vector which is based on an efficiently replicating variant of Semliki Forest virus [28]. mRNA was then synthesized from *Spe*I-linearized plasmid DNA through the use of SP6 RNA polymerase [28] and was used for the transfection of BHK-21 cells.

The amino acids next to the predicted cleavage site of the N-terminal propeptide of H3 –Lys^{-2}-Arg^{-1}– were changed to Asn-Ser with the U.S.E. mutagenesis kit (Pharmacia). The mutagenic primer was GCT GCT TGG G**AA CTC G**AG CCT TCC AG with the amino acid substitutions marked in bold. All constructions were confirmed by sequencing.

Transfection procedures

COS-1 cells were grown to subconfluence (80 %) in DMEM containing 10 % fetal bovine serum, glutamine and antibiotics (complete DMEM). After release with trypsin, the cells were washed with PBS supplemented with 10 mM Hepes and finally suspended in PBS/Hepes at a concentration of 24×10^6 cells/ml. Then 0.5 ml aliquots of the cell suspension were transferred into electroporation cuvettes (0.5 cm) and 15 μg, or 2×15 μg for co-transfections, of plasmid DNA was added, together with 50 μg of fish sperm DNA (Boehringer Mannheim, Germany). After electroporation at 500 μF and 0.3 kV (Bio-Rad; Hercules, CA, U.S.A.), the cells were immediately diluted into complete DMEM

and plated onto 3.5 cm Petri dishes in complete DMEM. The medium was changed after 4 h.

BHK-21 cells were grown to subconfluence (80 %) in Glasgow medium supplemented with 10 % tryptose phosphate broth, 5 % fetal bovine serum and 20 mM Hepes, pH 7.3 (complete medium). After release with trypsin, the cells were washed and finally suspended in PBS at a concentration of 10×10^6 cells/ml. About 2 μg of mRNA, obtained from transcription of the pSVF3-H3 vector, was then electroporated into 5×10^6 BHK-21 cells in 0.5 ml (two pulses of 0.85 kV, 25 μF). The cells were immediately diluted into complete medium and added to 6 cm Petri dishes. The medium was changed after 4 h.

Expression experiments

The day after the COS-1 cells had been transfected, the medium was replaced by serum- and methionine-free DMEM supplemented with glutamine, 1 mg/ml BSA and 2 μM methionine. [^{35}S]methionine (0.1 mCi/plate) was added 30 min later and the cells were labelled for 3–4 h. In some experiments the labelling was done in the presence of 2.5 mM deoxymannojirimycin (Sigma). The medium of the labelled cells was then collected and used for immunoprecipitation. Some samples were first treated with chondroitinase ABC as follows: 800 μl of medium was supplemented with 50 mM Tris/HCl, pH 8.0, 0.2 mM PMSF, 0.1 mM *N*-ethylmalemide, 1 μg/ml pepstatin and 5 m-units of chondroitinase ABC (Sigma) and incubated for 14 h at 37 °C. Alternatively, the immunocomplexes were suspended in 30 μl of the same buffer and incubated.

Approximately 20 h after transfection of the BHK-21 cells, the cells were labelled for 10 min with [^{35}S]methionine (0.1 mCi/plate). After different times of chase, the plates were placed on ice and the medium was collected. The cells were rinsed once with ice-cold PBS and 1 ml of solubilization buffer was added (20 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, 1 % Triton X-100, 5 mM EDTA, 1 % Trasylol (Bayer), 0.2 mM PMSF, 0.1 mM *N*-ethylmalemide and 1 μg/ml pepstatin). The solubilized cells were centrifuged at 9000 ***g*** for 5 min and the supernatant was collected and used for immunoprecipitation.

Immunoprecipitation

Antiserum against human α_1-antitrypsin (2 μl; Dakopatts, Glostrup, Denmark) was added to 400 μl of the cell extracts and 800 μl of the cell media. After 1 h of incubation at +4 °C, 100 μl of a 10 % (w/v) suspension of glutaraldehyde-fixed *Staphylococcus aureus* was added. The test tubes were rotated end-over-end for 45 min and were then centrifuged for 5 min at 9000 ***g***. The supernatants were collected and 5 μl of rabbit antibodies against rat serum proteins (Dakopatts), bikunin, PαI or H3 were added. After 16 h, 100 μl of 10 % *S. aureus* particles was added and the immunocomplexes were collected by centrifugation as described above. The pellets were washed once with 10 mM Tris/HCl, pH 7.5, containing 0.4 M NaCl, 1 % Triton X-100 and 5 mM EDTA, and once with 10 mM Tris/HCl, pH 7.5. The immune complexes were analysed by SDS/PAGE followed by fluorography.

Endo-β-*N*-acetylglucosaminidase H (EndoH) treatment

The pellets obtained from the immunoprecipitation were suspended in 15 μl of 50 mM sodium acetate, pH 5.6, containing 3 g/l SDS. After heating at 95 °C for 5 min, the samples were centrifuged at 9000 ***g*** for 5 min. The supernatants were collected and 10 μl of 50 mM sodium acetate, pH 5.6, containing 0.1 m-units of EndoH (Boehringer Mannheim, Germany) was added. After 6 h of incubation at 37 °C, sample buffer (20 μl) was added and the proteins were analysed by SDS/PAGE followed by fluorography.

RESULTS

Cloning and sequencing of cDNA for rat H3

We are using the rat to study the biosynthesis and function of bikunin-containing proteins. To be able to use rat H3 for expression experiments, we first cloned and sequenced its cDNA. To this end, a rat liver cDNA library was screened with a probe containing a 2 kb fragment of the cDNA for human H3. Twelve overlapping clones were obtained which were then used for the determination of the full-length sequence of the cDNA for rat H3; the sequence was submitted to the EMBL database and was given the accession number X83231. The sequence has an open reading frame of 2663 nucleotides encoding a protein of 887 amino acid residues with a molecular mass of 99 kDa (see Figure 1). The degrees of identity with the corresponding human protein on the amino acid and nucleotide levels are 82 and 81 % respectively. The corresponding values for mouse H3 [29] are 93 and 91 % respectively. Optimal alignment of the rat sequence with that of man required the introduction of a gap around amino acid residue 630, as previously described for the mouse proteins [29]. In general, the region between residues 610 and 740 contains more divergent sequences then the rest of the molecule.

Using the rules for the prediction of the cleavage site of a signal peptide [30], we found that this sequence is 21 amino acid residues long in rat H3. Comparison of the amino acid sequences deduced from the cDNA of the human heavy chains with those obtained by direct analysis of the isolated proteins has revealed that, in addition to the signal peptide, these polypeptides are synthesized with both N- and C-terminal extensions [21–23]. The cleavage sites on the N-terminal side are next to dibasic sequences and those on the C-terminal side are between an Asp and a Pro residue within the common sequence DPHFII. The corresponding sequences in rat H3 with the putative cleavage sites are shown in Figure 1. The molecular masses of the resulting polypeptides are 2.3, 69 and 27 kDa.

Like all other heavy chains sequenced so far, rat H3 has two cysteine residues separated by only two amino acid residues in the N-terminal section. There are two potential N-glycosylation sites (NXT/S) in the mature H3 and one in the C-terminal extension. With the assumption that these sites are substituted

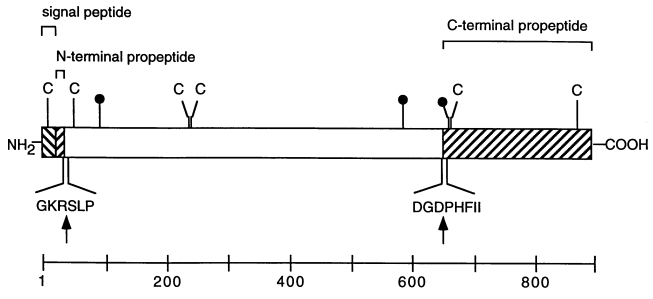

Figure 1 Schematic structure of rat H3

The nucleotide sequence of the cDNA for rat H3 was determined as described in the text and was found to code for a polypeptide 887 amino acids long (shown as a box). Cysteine residues and potential N-glycosylation sites are indicated with C and ●, respectively. The hatched regions indicate peptides released during intracellular transport; the amino acid sequences around two of the putative cleavage sites are also shown.

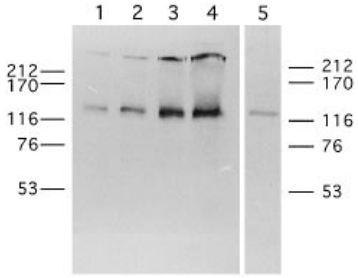

Figure 2 Detection of bikunin-containing proteins in rat serum

Different amounts of rat serum were subjected to SDS/PAGE and the proteins were transferred to a nitrocellulose membrane; lanes 1–5 contained 0.05, 0.1, 0.5, 2 and 2 μl of serum respectively. Detection was performed with antibodies against bikunin (lanes 1–4) and H3 (lane 5). The positions of reference proteins are shown on each side with their molecular masses in kDa.

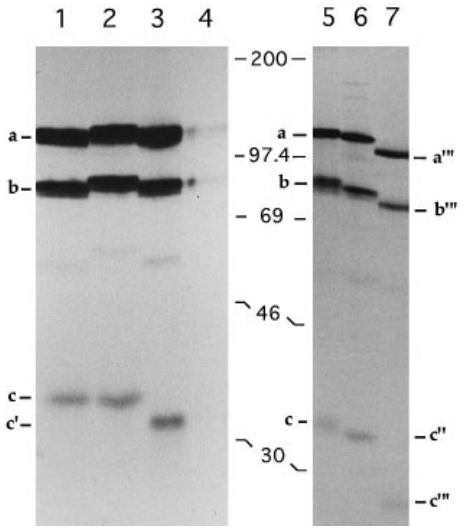

Figure 3 Expression of H3 in COS-1 cells

The cells were transfected with cDNA coding for wild-type H3 or for H3 with a mutated cleavage site for the N-terminal propeptide. The cells were then labelled with [^{35}S]methionine for 3 h. The media were collected and antibodies to rat serum proteins added. The immune complexes were analysed by SDS/PAGE followed by fluorography. The indicated bands are discussed in the text. Lanes 1, 3, 5, 6 and 7 show wild-type H3 and lane 2 shows the mutated form. Lane 4 shows a sample from mock-transfected cells. No reducing agent was added to the sample in lane 3. The sample in lane 6 was prepared from cells labelled in the presence of an inhibitor of carbohydrate processing. Lane 7 shows the same sample which was preincubated with EndoH before electrophoresis. The positions and molecular masses (in kDa) of ^{14}C-labelled reference proteins are shown in the centre.

and that the oligosaccharides are of the simple biantennary type, the molecular masses of the mature H3 and the C-terminal peptide can be estimated as 74 and 29 kDa respectively.

H3 is the heavy chain of rat PαI

We wanted to determine which of the bikunin-containing proteins of rat serum contains H3. Analysis of rat serum with SDS/PAGE, followed by immunostaining with an antibody against bikunin, showed two bands with apparent molecular masses of 230 and 125 kDa (Figure 2). Based on results obtained earlier with the human proteins [27], we conclude that these bands contain IαI and PαI respectively. Densitometric analysis showed that the amount of PαI was 9–10 times higher than that of IαI. We have previously shown that the total amount of bikunin in rat serum is 0.17 mg/ml, 98 % of which is in complex with other polypeptides [31]. Given a molecular mass of 99 kDa for PαI, the amount of PαI can be estimated to be 0.4 mg/ml; similar values have been reported for the concentration of IαI in human serum [32,33]. Antibodies specific for H3 were also used to probe the same material, which resulted in staining of PαI only (Figure 2, lane 5).

For a direct identification of the heavy chains of rat PαI and IαI, we isolated these proteins from serum, released the heavy chains from bikunin by treatment with high pH [27] and separated the polypeptides by SDS/PAGE. After transfer to a membrane, the heavy chains were subjected to N-terminal amino acid sequencing. The sequence obtained for the heavy chain of PαI , SLPEGVV, was identical with that predicted from our cDNA clone. The sequence obtained for the slower-migrating heavy chain of IαI, SLSEDDGEE, was similar to that of the human H2 [34]. The other chain yielded the sequence AVDTS, which is identical with the sequence from amino acid residues 2–6 of the H1 of human IαI. The latter polypeptide has been shown to have a blocked N-terminal amino acid residue [35]. It is possible that this is also the case for the rat protein and that the sequencing reaction therefore detected only a truncated form. Taken together, these data show that the chain compositions of the high-molecular-mass complexes of bikunin in rat are identical with those of man.

Expression of H3

As the first step in reconstituting the assembly of PαI, we expressed rat H3 in COS-1 cells and analysed the medium by immunoprecipitation followed by SDS/PAGE. Figure 3 (lane 1) shows that three labelled polypeptides with the apparent molecular masses 115, 82 and 33 kDa were detected by this procedure (denoted a, b and c respectively). We assume that these are the H3 precursor, H3 and the C-terminal propeptide respectively. Densitometric analysis of the bands indicated that 40–60 % of H3 was cleaved. We also wanted to determine whether secreted H3 lacked the N-terminal propeptide. To perform this analysis simply by SDS/PAGE we needed the uncleaved form of H3 as a reference material. With the assumption that the two basic amino acids next to the cleavage site (see Figure 1) were essential for cleavage, we mutated these to Asn and Ser and expressed the modified protein in COS-1 cells. Upon electrophoresis, this protein had a slightly higher apparent molecular mass than the wild-type form (Figure 3; cf. bands a and b in lanes 1 and 2). This result indicates that the N-terminal propeptide of wild-type H3 was efficiently cleaved in these cells. Furthermore, we found that the released C-terminal propeptide displayed a higher mobility under non-reducing conditions (lane 3, band c′) than under reducing conditions (lane 1, band c), implying that it contains a disulphide bridge.

We wanted to determine whether H3 and the C-terminal propeptide secreted by the COS-1 cells carried N-linked carbohydrates. The shift in electrophoretic mobility caused by treatment with EndoH is a simple way of detecting this type of carbohydrate on proteins. However, EndoH removes only early, mannose-rich oligosaccharides, whereas those on secreted proteins are usually resistant. To overcome this limitation, we blocked the conversion of the oligosaccharides to EndoH-resistant forms during intracellular transport, by adding deoxymannojirimycin to the medium; this compound inhibits a Golgi-specific mannosidase whose action is necessary for the maturation of N-linked oligosaccharides [36]. The polypeptides obtained

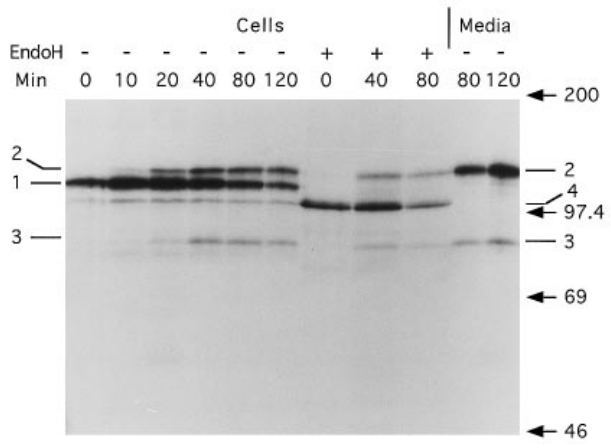

Figure 4 Pulse–chase analysis of the intracellular processing of rat H3 in BHK-21 cells

The cells were transfected with cDNA for H3, labelled with [^{35}S]methionine for 10 min and chased for the times indicated. Antibodies against rat serum proteins were added to the media and the solubilized cells. The immunoprecipitates were analysed by SDS/PAGE followed by fluorography. Some of the samples were treated with EndoH before electrophoresis, as indicated. The protein bands labelled 1–4 are discussed in the text.

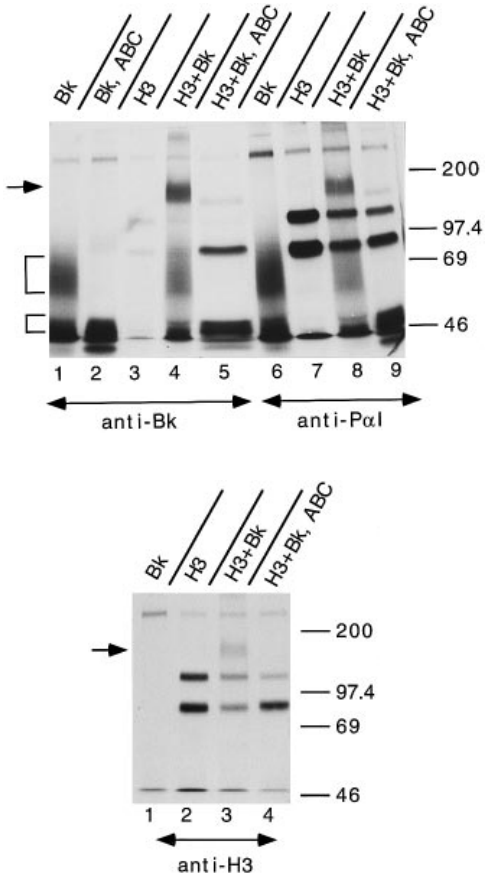

Figure 5 Co-expression of H3 and bikunin in COS-1 cells

COS-1 cells were transfected with cDNA for the bikunin precursor (Bk) and/or for H3 as indicated. The cells were then labelled for 60 min with [^{35}S]methionine. The media were collected and antibodies against bikunin, PαI or H3 were added as indicated. The immunoprecipitates were analysed by SDS/PAGE followed by fluorography. The upper and lower brackets indicate the bikunin precursor with and without chondroitin sulphate respectively. The arrow indicates the complex between bikunin and H3 that formed upon coexpression. The samples in lanes 2 and 9 (top panel) and lane 4 (bottom panel) were treated with chondroitinase ABC before immunoprecipation and the sample in lane 5 after immunoprecipitation.

from cells treated with deoxymannojirimycin had slightly higher mobilities than the normal forms, most evident for the C-terminal propeptide: cf. bands c and c″ (Figure 3, lanes 5 and 6). Such a difference in mobility is often seen between intra- and extra-cellular forms of glycoproteins and is an indication that maturation of the oligosaccharides has occurred [37]. Treatment with EndoH of the recombinant proteins secreted from cells incubated with deoxymannojirimycin increased their electrophoretic mobilities (lane 7, bands a‴, b‴ and c‴; apparent molecular masses 98, 79 and 27 kDa respectively). This result shows that both cleaved H3 and the C-terminal propeptide contain N-linked oligosaccharides.

We also ascertained the processing of H3 in a different cell line: BHK-21. Figure 4 shows a pulse–chase experiment with these cells. The primary translation product was a polypeptide with an apparent molecular mass of 112 kDa (band 1). (There was also a minor band of about 100 kDa, which may represent unglycosylated H3, see below). Upon chase, the apparent molecular mass of H3 increased to about 123 kDa (band 2), presumably due to the maturation of N-linked carbohydrates. Simultaneously, a faster migrating polypeptide was formed (band 3), which, based on its apparent molecular mass (85 kDa), probably represents H3 lacking the C-terminal propeptide. Treatment of the same samples with EndoH decreased the apparent molecular mass of band 1 to 102 kDa (band 4), whereas bands 2 and 3 were unaffected. Since EndoH resistance is acquired in the Golgi complex and there was no trace of a sensitive form of band 3, these results indicate that cleavage of H3 occurs in the Golgi. The relative amount of cleaved H3 (band 3) was markedly lower than in the medium of the COS-1 cells; the C-terminal propeptide could therefore not be detected in these samples.

Coupling of bikunin and H3

Having established that transfection of COS-1 cells with cDNA for rat H3 would yield proper processing and secretion of the protein, we transfected COS-1 cells with cDNA for the bikunin precursor. Electrophoretic analysis of the secreted proteins that were isolated with antibodies against bikunin yielded two bands, with apparent molecular masses of about 70 and 47 kDa [Figure 5 (top), lane 1; upper and lower brackets respectively]. These values agree with those previously found for the bikunin precursor with and without chondroitin sulphate respectively [19]. Consistent with this interpretation, only the upper band was sensitive to chondroitinase treatment [Figure 5 (top), lane 2]. Furthermore, Bratt et al. [38] have previously found that there is little cleavage of the bikunin precursor in COS-1 cells. (Mature bikunin and bikunin lacking the chondroitin sulphate chain run as proteins of 42 and 28 kDa respectively upon SDS/PAGE [19]). With cells transfected with cDNA for H3, no specific bands were detected with the bikunin antibodies [Figure 5 (top), lane 3]. The medium of cells transfected with cDNA for both bikunin and H3, analysed with the same antibodies [Figure 5 (top), lane 4], yielded the bikunin precursor as well as a larger protein (indicated with an arrow). The apparent molecular mass of the latter protein (140 kDa) indicated that it was formed by linkage of cleaved H3 (82 kDa) and the bikunin precursor (70 kDa), i.e. that the protein was PαI containing α_1-microglobulin. Further

support for this assumption came from the findings that the protein was sensitive to treatment with chondroitinase ABC and that the treatment resulted in the appearance of a band with an apparent molecular mass slightly higher than that of cleaved H3 [Figure 5 (top), lane 5]. The fact that the band formed upon chondroitinase treatment had a higher molecular mass than secreted H3 could be due to incomplete degradation. Analysis of the medium of cells transfected with cDNA for bikunin only [Figure 5 (top), lane 6] with antibodies against PαI (which reacted with both H3 and bikunin) yielded the bikunin precursor with and without chondroitin sulphate, as shown in lane 1. When the cells were transfected with cDNA for H3 only [Figure 5 (top), lane 7], cleaved and uncleaved H3 were the major bands. Cells transfected with cDNA for both H3 and bikunin [Figure 5 (top), lane 8] yielded the bikunin precursor, the two H3 bands as well as the 140 kDa band. Treatment of this material with chondroitinase ABC removed the 140 kDa band as well as the chondroitin sulphate-containing bikunin precursor [Figure 5 (top), lane 9]).

To test more directly whether the 140 kDa band contained H3, we prepared an antiserum specific for the heavy chains. The antibodies in this antiserum did not recognize bikunin [Figure 5 (bottom), lane 1], whereas they bound to both forms of H3 [Figure 5 (bottom), lane 2]. When the same antibodies were added to the medium of cells co-expressing bikunin and H3, cleaved and uncleaved H3 were detected as well as the 140 kDa protein [Figure 5 (bottom), lane 3]. If the same material was treated with chondroitinase ABC before electrophoresis, the 140 kDa band did not appear [Figure 5 (bottom), lane 4]. Assuming that the coupling reaction in the COS-1 cells is the same as in hepatocytes, only cleaved H3 would be linked to bikunin, in which case the relative amount of free, cleaved H3 would decrease upon cotransfection with bikunin cDNA. Our results do not clearly show such a relationship, possibly because the degree of cleavage of H3 varied between different transfections, making comparisons difficult. Alternatively, the presence of the bikunin precursor may induce some cleavage, resulting in an apparently unchanged ratio of uncleaved to cleaved H3.

DISCUSSION

The bikunin-containing proteins PαI and IαI are the only proteins known in which a glycosaminoglycan forms a covalent link between polypeptides. So far, only hepatocytes have been shown to produce proteins with this structure. In the present study we show that when bikunin and H3, the two polypeptides of PαI, are expressed in COS-1 cells, a covalent chondroitinase-sensitive complex between bikunin and H3 is formed. Whether the two polypeptides are linked by an ester bond, as demonstrated for PαI and IαI isolated from serum, remains to be seen. The finding that COS-1 cells have the capacity to form a chondroitin sulphate-containing link, suggests that non-hepatic cells may produce proteins with this or similar structures. In this context it is interesting to note that mRNAs for H2 and H3 (but not for bikunin) have been detected in mouse brain [29].

One characteristic feature of the heavy chains of the bikunin-containing proteins is that they are synthesized with a C-terminal extension. This propeptide is released during intracellular transport, apparently through a scission between an Asp and a Pro residue. The sequence around the cleavage site, DPHFII, seems to be the same for all heavy chains studied so far [29]. Interestingly, there is no known proteinase with specificity for this site. Cleavage of the heavy chains is apparently not autocatalytic, as judged by the fact that H3 is secreted in uncleaved form from the human hepatoma cell line HepG2 [23]. It has been suggested that release of the C-terminal propeptide might be mediated by the chondroitin sulphate chain of bikunin [4]. Our finding that H3 expressed alone in COS-1 cells is efficiently cleaved indicates that this is not the case. However, it cannot be ruled out that the bikunin molecule does affect the cleavage reaction *in vivo*. We have previously shown that in rat hepatocytes the α_1-microglobulin–bikunin precursor is rapidly coupled to the heavy chains as it acquires its chondroitin sulphate chain [19]. After the coupling reaction, it takes 5–10 min before the precursor is proteolytically cleaved and α_1-microglobulin is released. Thus cleavage of the heavy chains occurs before cleavage of the bikunin precursor. Whether this temporal difference reflects a difference in the subcellular distribution of the cleaving enzymes remains to be seen.

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REFERENCES

1 Swaim, M. W. and Pizzo, S. V. (1988) Biochem. J. **254**, 171–178
2 Gebhard, W., Hochstrasser, K., Fritz, H., Enghild, J. J., Pizzo, S. V. and Salvesen, G. (1990) Biol. Chem. Hoppe-Seyler **371**, 13–22
3 Diarra-Mehrpour, M., Bourguignon, J., Sesboüé, R., Mattei, M. G., Passage, E., Salier, J. P. and Martin, J. P. (1989) Eur. J. Biochem. **197**, 147–154
4 Enghild, J. J., Salvesen, G., Hefta, S. A., Thogersen, I. B., Rutherfurd, S. and Pizzo, S. V. (1991) J. Biol. Chem. **266**, 747–751
5 Morelle, W., Capon, C., Balduyck, M., Sautiere, P., Kouach, M., Michalski, C., Fournet, B. and Mizon, J. (1994) Eur. J. Biochem. **221**, 881–888
6 Ødum, L. (1990) Biol. Chem. Hoppe-Seyler **371**, 1153–1158
7 Castillo, G. M. and Templeton, D. M. (1993) FEBS Lett. **318**, 292–296
8 McKeehan, W. L., Sakagami, Y., Hoshi, H. and McKeehan, K. A. (1986) J. Biol. Chem. **261**, 5378–5383
9 Perry, J. K., Scott, G. K. and Tse, C. A. (1994) Biochim. Biophys. Acta **1221**, 145–152
10 Kobayashi, H., Fuije, M., Shinohara, H., Ohi, H., Sugimura, M. and Terao, T. (1994) Int. J. Cancer **57**, 378–384
11 Chen, L., Mao, S. J. and Larsen, W. J. (1992) J. Biol. Chem. **267**, 12380–12386
12 Chen, L., Mao, J. T., McLean, L. R., Powers, R. W. and Larsen, W. J. (1994) J. Biol. Chem. **269**, 28282–28287
13 Blom, A., Pertoft, H. and Fries, E. (1995) J. Biol. Chem. **270**, 9698–9701
14 Zhao, M., Yoneda, M., Okashi, Y., Kurono, S., Iwata, H., Ohnuki, Y. and Kimata, K. (1995) J. Biol. Chem. **270**, 26657–26663
15 Yoneda, M., Suzuki, S. and Kimata, K. (1990) J. Biol. Chem. **265**, 5247–5257
16 Vetr, H. and Gebhard, W. (1990) Biol. Chem. Hoppe-Seyler **371**, 1185–1196
17 Lindqvist, A., Bratt, T., Altieri, M., Kastern, W. and Akerstrom, B. (1992) Biochim. Biophys. Acta **1130**, 63–67
18 Åkerström, B. and Lögdberg, L. (1990) Trends Biochem. Sci. **15**, 240–243
19 Sjöberg, E. M. and Fries, E. (1992) Arch. Biochem. Biophys. **295**, 217–222
20 Bratt, T., Olsson, H., Sjöberg, E. M., Jergil, B. and Akerstrom, B. (1993) Biochim. Biophys. Acta **1157**, 147–54
21 Gebhard, W., Schreitmuller, T., Hochstrasser, K. and Wachter, E. (1989) Eur. J. Biochem. **181**, 571–576
22 Bourguignon, J., Diarra, M. M., Thiberville, L., Bost, F., Sesboue, R. and Martin, J. P. (1993) Eur. J. Biochem. **212**, 771–776
23 Thogersen, I. B. and Enghild, J. J. (1995) J. Biol. Chem. **270**, 10700–10709
24 Heron, A., Borghi, H., Calle, A., Bourguignon, J., Diarra-Mehrpour, M., Martin, J. P. and Sesboue, R. (1995) Cell Biol. Int. **19**, 593–602
25 Berthold, H., Frorath, B., Scanarini, M., Abney, C. C., Ernst, B. and Northemann, W. (1992) Biotechnol. Lett. **14**, 245–250
26 Sjöberg, E., Blom, A., Sjöquist, M., Wolgast, M., Alston-Smith, J. and Fries, E. (1995) Biochem. J. **308**, 881–887
27 Enghild, J. J., Thogersen, I. B., Pizzo, S. V. and Salvesen, G. (1989) J. Biol. Chem. **264**, 15975–15981
28 Liljeström, P., Lusa, S., Huylebroeck, D. and Garoff, H. (1991) J. Virol. **66**, 4107–4113

29 Chan, P., Risler, J. L., Raguenez, G. and Salier, J. P. (1995) Biochem. J. **306**, 505–512
30 von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
31 Slota, A., Sjöquist, M., Wolgast, M., Alston-Smith, J. and Fries, E. (1994) Biol. Chem. Hoppe-Seyler **375**, 127–133
32 Salier, J. P., Sesboüé, R., Vercaigne, D., Bourguignon, J. and Martin, J. P. (1983) Anal. Biochem. **133**, 336–343
33 Liony, C., Sesboué, R., Manchon, N. D., Bercoff, E., Delzant, G., Martin, J. P. and Bourreille, J. (1991) Presse Med. **20**, 203–206
34 Gebhard, W., Schreitmuller, T., Hochstrasser, K. and Wachter, E. (1988) FEBS Lett. **229**, 63–67
35 Malki, N., Balduyck, M., Maes, P., Capon, C., Mizon, C., Han, K. K., Tartar, A., Fournet, B. and Mizon, J. (1992) Biol. Chem. Hoppe-Seyler **373**, 1009–1018
36 Gross, V., Steube, K., Tran-Thi, T. A., McDowell, W., Schwarz, R. T., Decker, K., Gerok, W. and Heinrich, P. C. (1985) Eur. J. Biochem. **150**, 41–46
37 Fries, E. and Lindström, I. (1986) Biochem. J. **237**, 33–39
38 Bratt, T., Cedervall, T. and Åkerström, B. (1994) FEBS Lett. **354**, 57–61

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