Identification of Placental Transforming Growth Factor-β and Bikunin Metabolites as Contaminants of Pharmaceutical Human Chorionic Gonadotrophin Preparations by Proteomic Techniques*

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A contaminant protein complex found in pharmaceutical urinary human chorionic gonadotrophin preparations is reported to have anti-human immunodeficiency virus-associated Kaposi's sarcoma activity. The aim of this study was to isolate and characterize this protein complex by proteomic approaches. Size exclusion chromatography was used in the isolation of these human chorionic gonadotrophin-associated fragments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the presence of a protein complex that dissociated into two protein bands under reducing conditions. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of this complex showed three polypeptides at ~6.2, 11.4, and 15.8 kDa. Peptide mass mapping and N-terminal amino acid sequencing identified two polypeptides as metabolites of placental transforming growth factor-β (11.4 kDa) and bikunin (15.8 kDa). Subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of the anti-human immunodeficiency virus-associated Kaposi's sarcoma active preparations CG-10 (Sigma), Pregnyl (Organon), and Profasi (Serono) revealed the presence of metabolites of placental transforming growth factor-β in all three; no other non-human chorionic gonadotrophin-related protein species were observed in these preparations. Our findings present evidence that urinary human chorionic gonadotrophin preparations are contaminated with metabolites of placental transforming growth factor-β, which may have transforming growth factor-β agonist actions, and metabolites of bikunin, which is a protease inhibitor. In combination these molecules may be responsible for the anti-human immunodeficiency virus-associated Kaposi's sarcoma activity demonstrated for these urinary human chorionic gonadotrophin preparations. ***Molecular & Cellular Proteomics 4:984–992, 2005.***

Human chorionic gonadotrophin (hCG)[1] is the principal hormonal product of the conceptus and is responsible for signaling maternal recognition of pregnancy in humans. More recently it has been reported to inhibit human immunodeficiency virus (HIV) replication in maternal blood cells during pregnancy and transmission from lymphocytes to trophoblasts (1). It was later proposed that the antiviral activity of hCG in blood cells was determined by its free β-subunit (hCGβ) (2). The anti-HIV effects of hCG and hCGβ have also been noted in HIV-associated Kaposi's sarcoma (KS) (3–7). KS usually presents as lesions consisting of aggregates of spindle-shaped cells interspersed with endothelium-lined channels, which remain confined to the subcutaneous layer of the skin. Human hepatitis virus-8 is the main causative agent of KS, but HIV infection results in a completely distinct manifestation; lesions arise all over the body but particularly affect the face and the moist mucosa of the mouth and nose (8). HIV-induced KS is the most common tumor of patients infected with the virus, and the risk of developing the sarcoma is increased 20,000-fold in acquired immunodeficiency syndrome sufferers (8). The anti-HIV-KS activity of hCG was initially noted as part of an investigation into the significance of the male gender bias of HIV-associated KS: Kaposi observed a bias of 15:1 toward male presentation for this condition in his initial study (for a review, see Ref. 9). Lunardi-Iskandar *et al.* (4) at the NCI, National Institutes of Health

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[1] The abbreviations used are: hCG, human chorionic gonadotrophin; HAF, human chorionic gonadotrophin-associated fragments; hCGα, human chorionic gonadotrophin α-subunit; hCGβ, human chorionic gonadotrophin β-subunit; hCGβcf, human chorionic gonadotrophin β core fragment; HIV, human immunodeficiency virus; KS, Kaposi's sarcoma; m-bik, bikunin metabolite; MIC-1, macrophage inhibitory cytokine-1; m-pTGF-β, placental transforming growth factor-β metabolite; nESI, nano-ESI; pTGF-β, placental transforming growth factor-β; TATI, tumor-associated trypsin inhibitor; TGF-β, transforming growth factor-β.

(Bethesda, MD) noticed that mice with induced KS who then became pregnant went into tumor remission. They went on to describe how preparations of hCG and hCGβ "killed KS cells *in vitro* and *in vivo*, apparently by apoptosis." The effect was initially attributed to luteinizing hormone/hCG receptor activation, but the mouse does not possess a chorionic gonadotrophin β gene or express an equivalent gonadotrophin during pregnancy (10). Various clinical trials were carried out; most of them used crude hCG preparations. In the majority of studies, hCG administration resulted in various degrees of KS lesion remission (11–17). In response to this, Krown (18) commented on the variation in the quality of hCG preparations; the free subunits of hCG and the urinary metabolite of hCG, hCGβ core fragment (hCGβcf), were common contaminants. Rabkin *et al.* (19) suggested that a contaminant factor present in pregnancy urine hCG must explain the tumor regression in pregnant mice. Albini *et al*. (20) concluded that the active compound was hCGβcf. However, others fractionating anti-HIV-KS active hCG preparations suggested that the anti-HIV-KS activity was due to small contaminating molecules that eluted close to hCGβcf on size exclusion columns (21). Various low molecular weight components have now been isolated from neat pregnancy urine and urinary hCG preparations with anti-HIV-KS activity. These have been variously described as antiviral lysozyme C and antiviral RNases (22–24). However, none have been unequivocally identified and are best described as an unidentified protein complex termed "hCG-associated fragments" (HAF) (25, 26).

We have previously isolated and purified a contaminant complex of the urinary hCG preparation CG-10 (Sigma) that appeared to co-elute in our isolation of hCGβcf (27). We now report the characterization of this protein complex by SDS-PAGE, N-terminal sequencing, and mass spectrometry and confirm its presence in urinary hCG preparations found to have anti-HIV-KS activity.

EXPERIMENTAL PROCEDURES

Reagents—Three clinical grade hCG preparations were used: CG-10 (Sigma), Pregnyl (Organon, Cambridge, UK), and Profasi (Serono, Feltham, UK). The MALDI-TOF MS grade matrices α-cyano-4-hydroxycinnamic and 2,5-dihydroxybenzoic acids were obtained from Fluka (Poole, UK). All other chemicals were obtained from Merck unless otherwise stated.

Size Exclusion Chromatography—HAF was isolated from CG-10 by size exclusion chromatography on a Sephadex G-100 column (Amersham Biosciences) as described previously (27). Briefly the material was dissolved in 0.01 M phosphate buffer saline prior to chromatography. The column was then eluted with the above buffer.

Assays—Total protein was measured by using the Bradford dye (0.5 mg/ml Coomassie Blue G in 25% ethanol, 42.5% concentrated phosphoric acid). Absorbance was read at 570 nm by using an EL311SX microtiter plate reader (Anachem, Luton, UK).

The S781 polyclonal antibody was used as the capture antibody against intact hCG, whereas the S752 monoclonal antibody was used as the capture antibody against free hCGβ (27). The M107 monoclonal antibody (27) was conjugated to horseradish peroxidase (Dako, Ely, UK) and was subsequently used as the detection antibody in both assays.

The INN112 monoclonal antibody was used as a capture antibody against the β_{13} epitope on hCGβcf; the INN112 antibody was a gift from Professor Peter Berger (Institute for Biomedical Aging Research, Innsbruck, Austria). The S504 polyclonal antibody (27) was used as a primary detection antibody for hCGβcf, and a donkey anti-sheep-horseradish peroxidase monoclonal antibody (Jackson Immunoresearch Inc., West Grove, PA) was used as a secondary detection antibody.

SDS-PAGE—One-dimensional SDS-PAGE was performed using 20% acrylamide separating gels and 5% stacking gels. Samples were denatured for 5 min at 100 °C prior to running under reducing and non-reducing conditions. Electrophoresis was performed in Tris-glycine buffer at a constant current of 15 mA. Gels were stained overnight with 25% Coomassie Blue (w/v) in 50% methanol, 10% acetic acid and destained with 50% methanol, 10% acetic acid.

In Situ Reduction, Alkylation, and Digestion—Reduction and alkylation of the stained proteins were done with dithiothreitol and iodoacetamide as described previously (28). Briefly stained protein bands were excised from gels and were further destained with 25 mM ammonium bicarbonate, 50% acetonitrile and reduced and alkylated with 10 mM dithiothreitol and 55 mM iodoacetamide. Tryptic digestion was carried out at a concentration of 12.5 ng/μl trypsin (Sigma) in a solution of 50 mM ammonium bicarbonate, 5 mM calcium chloride.

MALDI-TOF MS—A sample preparation protocol using MALDI target plates with a 600-μm hydrophilic anchor (AnchorChip™ 600, Bruker Daltonics, Coventry, UK) was used (29). All protein preparations and in-gel tryptic digests were desalted using C_{18} ZipTips (Millipore). An aliquot of the sample was acidified with 1 μl of 1% aqueous trifluoroacetic acid to enhance peptide binding on the C_{18} resin. Conditioning, loading, and washing steps were done according to the manufacturer's recommendation. Proteins and peptides were eluted from the C_{18} bed with 2 μl of acetonitrile, 0.1% aqueous trifluoroacetic acid (50:50, v/v) directly onto either a thin layer of α-cyano-4-hydroxycinnamic acid crystals formed from 0.3 μl of a 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid (acetone, 0.1% aqueous trifluoroacetic acid; 97:3) or a thin layer of 2,5-dihydroxybenzoic acid crystals formed from 0.5 μl of a 5 mg/ml solution of 2,5-dihydroxybenzoic acid (acetonitrile, 0.1% aqueous trifluoroacetic acid; 1:2). After drying, the α-cyano-4-hydroxycinnamic acid-containing preparations were recrystallized with 0.3 μl of ethanol, acetone, 0.1% aqueous trifluoroacetic acid (6:3:1).

MALDI-TOF mass spectra were acquired on an Autoflex (Bruker Daltonics, Coventry, UK) operated in the positive ion mode using a nitrogen laser emitting at 337 nm. Protein mass spectra were acquired in the linear mode, whereas digest spectra were recorded in reflectron mode for enhanced mass resolution. To avoid detector saturation low mass material was deflected. The detector voltage was 1.75 kV. All other parameters were set for an optimized mass resolution. Usually the mass spectra from 200 laser shots were averaged. Linear MALDI-TOF mass spectra were externally calibrated using the signals from bovine insulin, bovine ubiquitin, equine cytochrome *c*, and bovine α lactalbumin. MALDI spectra of tryptic digests were internally mass-corrected using trypsin autolysis fragments.

PSD MALDI-TOF MS—For PSD MALDI analysis, the pulsed ion deflector on the Autoflex was set to allow the selection of the precursor ion in a $\pm$5-Da mass window. Pulsed ion extraction of 80 ns was used for enhanced spectral resolution. The reflector voltage was stepped from a maximum of 20 kV depending on the mass of the precursor ion down to 0.59 kV in a maximum of 18 segments to focus metastable fragments on the detector. The spectra obtained at each reflector voltage setting were pasted together in XMASS™ using a calibration file from the PSD fragments of the adrenocorticotropic hormone clip 18–39. The mass assignment of fragments was performed in XMASS™ using an annotation macro for PSD spectra.

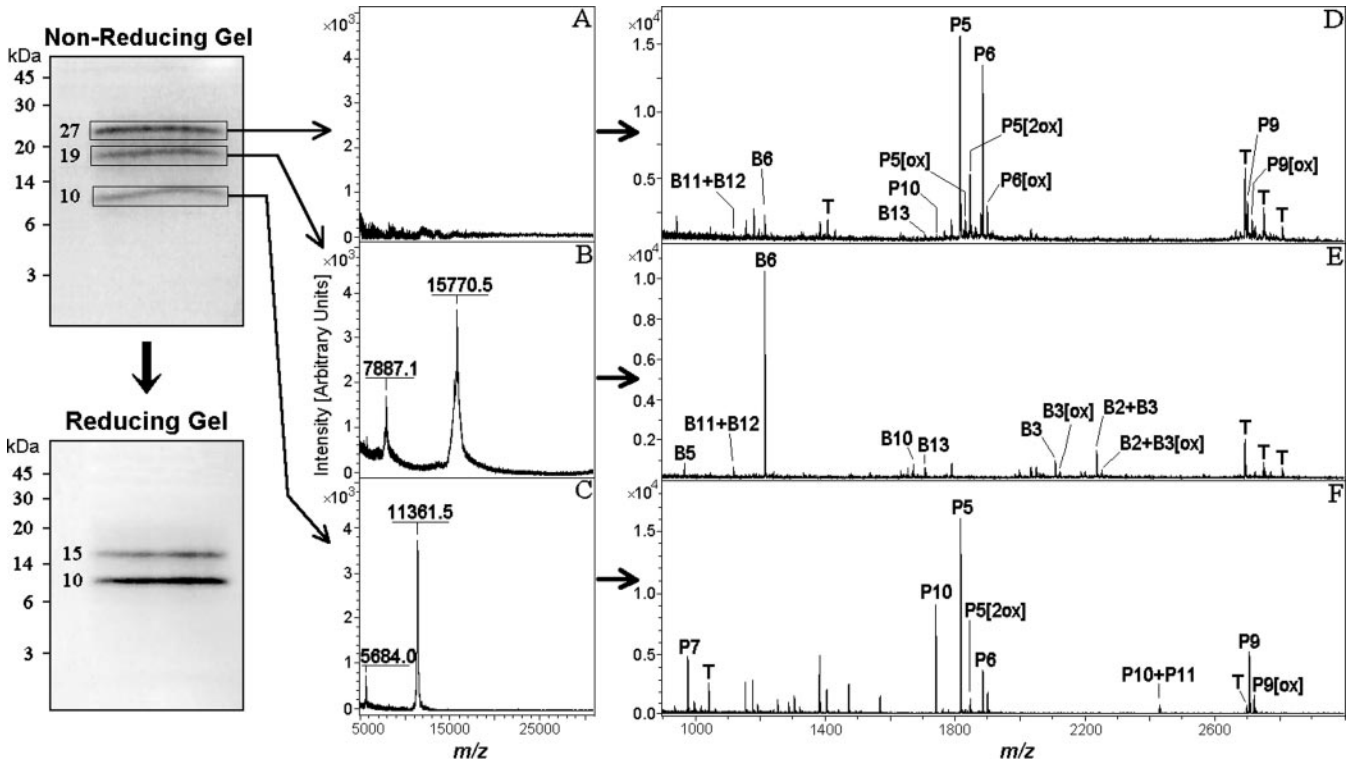

FIG. 1. **SDS-PAGE and MALDI-TOF MS of HAF preparation.** SDS-PAGE was performed under non-reducing and reducing conditions with *boxed areas* indicating the excised bands that were subsequently subjected to MALDI-TOF MS. *A–C*, eluted intact proteins as determined by MALDI-TOF MS. *D–F*, MALDI-TOF MS of tryptic digests with all identified signals being labeled. The coding for the mass labels can be found in Table I. Identical results were obtained with polypeptides eluted from reducing SDS-polyacrylamide gels (data not shown). *P*, m-pTGF-β fragments; *B*, m-bik fragments; *ox*, oxidation; *T*, trypsin autolysis fragments.

Nano (n)-ESI-MS—The peptide samples were desalted using C_{18} ZipTips as described above with the exception that formic acid was used instead of trifluoroacetic acid as an ion-pairing agent. The samples were directly infused into a LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) using PicoTip nanocapillaries (New Objective, Woburn, MA). Each ion species was isolated for an MS^2 experiment using an isolation width of 2–3 mass units, and the normalized collision energy was set between 30 and 40%.

N-terminal Sequencing by Edman Degradation—N-terminal sequences were provided as a service by Dr. J. Keen of the protein sequencing facility at the University of Leeds using a Procise 494 gas-phase/liquid-pulse sequencer (Applied Biosystems, Foster City, CA).

Carbohydrate Staining—To identify carbohydrates after SDS-PAGE, a glycoprotein detection kit (Sigma) was used in accordance with the procedures recommended by the manufacturer.

RESULTS

Isolation of HAF—In a previous study we fractionated protein molecules from the urinary hCG preparation CG-10 (Sigma) on a Sephadex G-100 column where hCG eluted first and hCGβcf appeared in later fractions. An unidentified protein complex (HAF) eluted closely to hCGβcf (data shown in previous publication) (27). Those fractions were pooled together and assayed for total protein (530 μg/ml), hCG (not detected, <1% cross-reactivity), hCGβ (not detected, <1% cross-reactivity), and hCGβcf (1,200 pmol/ml, ~2% cross-reactivity). SDS-PAGE of HAF showed protein bands at 27, 19, and 10 kDa under non-reducing conditions; under reducing conditions two bands were observed at 15 and 10 kDa (Fig. 1).

Molecular Identification of HAF—Polypeptides were eluted from individual gel bands produced under both non-reducing and reducing conditions and subjected to MALDI-TOF MS. Analysis of intact HAF components eluted from the non-reducing SDS-PAGE bands showed no MALDI signals for the 27-kDa gel band, two signals at *m/z* 15,770.5 (singly charged ion) and 7,887.1 (doubly charged ion) for the 19-kDa band, and two signals at *m/z* 11,361.5 (singly charged ion) and 5,684.0 (doubly charged ion) for the 10-kDa band (Fig. 1, *A–C*). In-gel tryptic digestion of the separated HAF components on non-reducing SDS-polyacrylamide gels followed by MALDI-TOF MS and a Mascot data base search (www.matrixscience.com) identified two polypeptides included within the 27-kDa gel band that were derived from placental transforming growth factor-β (pTGF-β) and bikunin and will be referred to here as metabolite (m)-pTGF-β and metabolite-bikunin (m-bik), respectively (Fig. 1*D*). Moreover the 19-kDa gel band contained only m-bik (~15.8 kDa with MALDI-TOF MS) (Fig. 1*E*), whereas the 10-kDa gel band contained m-pTGF-β (~11.4 kDa by MALDI-TOF MS) (Fig. 1*F*). Details of the tryptic mass mapping can be seen in Table I, and Fig. 2

TABLE I
Identification of tryptic fragments of HAF

HAF tryptic fragments shown in Fig. 1 were identified by using the Mascot search engine. P signals were obtained from m-pTGF-β, and B signals were obtained from m-bik. The N-terminal sequences of m-bik and m-pTGF-β as shown in Table II are bold.

Peptide no.	Range	Amino acid sequence	$[M + H]^+$		Mass accuracy
			Calculated	Observed	
					ppm
m-bik					
B1	1–3	**VTK**	347.23		
B2	4–4	**K**	147.11		
B3	5–23	**EDSCQL**GYSAGPCMGMTSR	2,106.83	2,106.83	0
B4	24–52	YFYNGTSMACETFQYGGCMGNGNNFVTEK	3,347.35		
B5	53–59	ECLQTCR	966.41	966.42	+10
B6[a]	60–70	TVAACNLPIVR	1,213.67	1,213.69	+16
B7	71–74	GPCR	489.22		
B8	75–86	AFIQLWAFDAVK	1,408.76		
B9	87–88	GK	204.13		
B10	89–103	CVLFPYGGCQGNGNK	1,670.74	1,670.71	−18
B11	104–108	FYSEK	673.32		
B12	109–111	ECR	464.19		
B13[a]	112–126	EYCGVPGDGDEELLR	1,708.75	1,708.77	+12
B14	127–129	FSN	367.16		
B2[b] B3	4–23	KEDSCQLGYSAGPCMGMTSR	2,234.93	2,234.92	−4
B11[b] B12[a]	104–111	FYSEKECR	1,118.49	1,118.51	+18
m-pTGF-β					
P1	1–2	**AR**	246.16		
P2	3–13	**NGDHCPLG**PGR	1,179.53		
P3	14–16	CCR	495.18		
P4	17–21	LHTVR	625.38	625.37[c]	+16
P5[a]	22–37	ASLEDLGWADWVLSPR	1,814.91	1,814.90	−6
P6[a]	38–53	EVQVTMCIGACPSQFR	1,882.87	1,882.89	+11
P7	54–62	AANMHAQIK	983.51	983.50	−10
P8	63–67	TSLHR	613.34	613.31[c]	−49
P9[a]	68–91	LKPDTVPAPCCVPASYNPMVLIQK	2,698.34	2,698.35	+4
P10[a]	92–107	TDTGVSLQTYDDLLAK	1,739.87	1,739.88	+6
P11	108–112	DCHCI	704.25	704.26[c]	+14
P10[b] P11	92–112	TDTGVSLQTYDDLLAKDCHCI	2,425.10	2,425.09	−4

[a] Tryptic peptides were also observed in the 27-kDa gel band from non-reducing SDS-polyacrylamide gels (Fig. 1*D*).
[b] Indicates a missed cleavage.
[c] Low molecular weight ions not shown in Fig. 1.

FIG. 2. **Amino acid sequences of m-bik and m-pTGF-β.** *A*, m-bik (129 amino acids); *B*, m-pTGF-β (112 amino acids). *Numbers* indicate amino acid positions on the precursor proteins. *Gray areas* represent tryptic peptides identified in Table I. *Italics*, N-terminal sequence; *, *N*-linked glycosylation site.

shows the full amino acid sequences of m-bik and m-pTGF-β. Identical results with the 19- and 10-kDa gel bands under non-reducing conditions were obtained from polypeptides eluted from reducing SDS-polyacrylamide gel bands at 15 and 10 kDa, respectively (data not shown).

PSD MALDI-TOF and nESI-MS were performed with some selected tryptic fragments to confirm peptide identifications. For example, PSD MALDI-TOF MS confirmed the presence of m-bik within the 19-kDa (non-reducing SDS-PAGE) and 15-kDa (reducing SDS-PAGE) gel bands by generating characteristic fragmentation of the major peptide ion at *m/z* 1,213.6 (Fig. 3). Furthermore nESI-MS confirmed the identity of some tryptic peptides of m-pTGF-β within the 10-kDa gel bands (non-reducing and reducing SDS-PAGE), for example at *m/z* 1,814.9 (Fig. 4).

To determine the N-terminal position of the HAF components, Edman degradation experiments were performed on polypeptides eluted from reducing SDS-polyacrylamide gels.

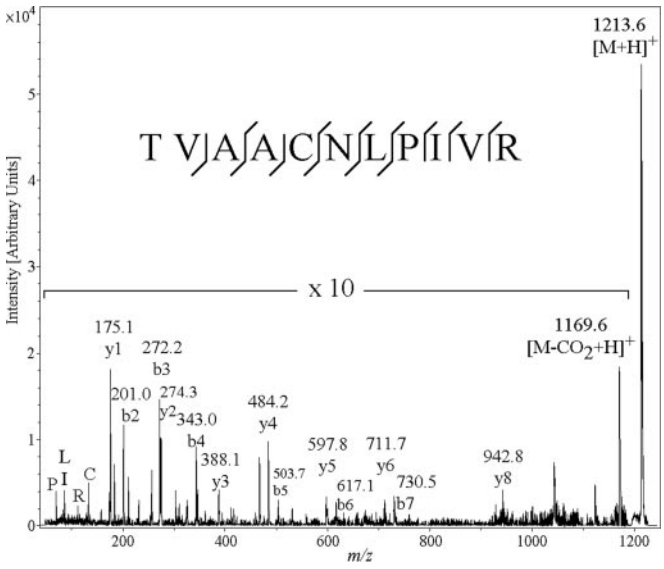

FIG. 3. **PSD MALDI-TOF MS of m-bik.** The 1,213.6-Da m-bik tryptic peptide B6 (Table I) was selected for PSD MALDI-TOF MS. Only the y and b ion series are labeled, and the ion coverage is indicated in the sequence string at the *top* of the figure.

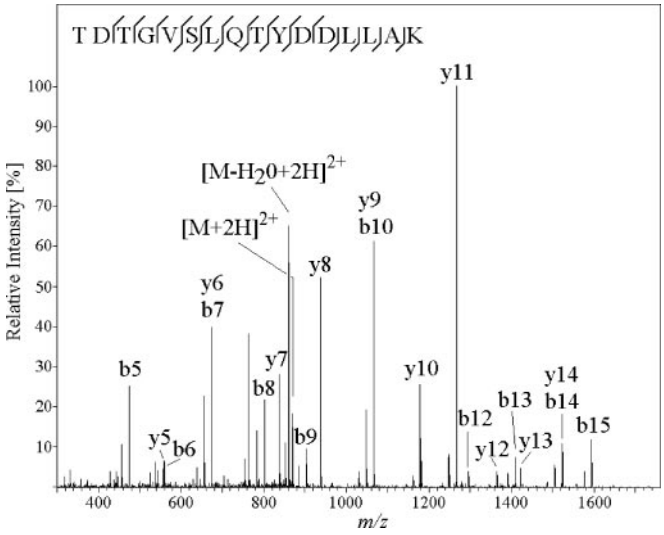

FIG. 4. **nESI-MS of m-pTGF-β.** The 1,814.9-Da m-pTGF-β tryptic peptide P5 (Table I) was selected for nESI-MS. The sequence at the *top* of the figure indicates the ion coverage for the peptide. Only the y and b ions are labeled in the spectrum.

The obtained N-terminal sequence tags were subjected to a protein BLAST search (www.ncbi.nlm.gov/BLAST) for short and nearly exact matches in protein and gene data bases. They generated significant sequence alignments with bikunin and pTGF-β for polypeptides eluted from reducing SDS-polyacrylamide bands at 15 and 10 kDa, respectively (Table II).

Carbohydrate Detection in HAF Preparation—Both bikunin and pTGF-β are known to contain carbohydrate structures (30, 31). Their presence on both m-bik and m-pTGF-β was investigated, although carbohydrate moieties were not expected to be found on m-pTGF-β (see "Discussion"). Carbohydrates were detected on SDS-PAGE bands that were run under non-reducing conditions. The SDS-PAGE protein band corresponding to m-bik at 19 kDa stained positively for carbohydrates, whereas all other bands were negative (Fig. 5). Tryptic digest MALDI-TOF analysis of m-bik resulted in an incomplete map of bikunin structure between amino acids 224 and 352 (Fig. 2*A*). In particular, a sequence spanning amino acids 247–275 (which includes the glycosylation site Asn^{250}) was not observed in the spectrum. This could indicate that glycosylation structures are still present and therefore contribute to the overall mass of m-bik (15.8 kDa). Further studies are necessary to determine the entire structure. This contrasts with m-pTGF-β in which almost the entire structure between amino acids 197 and 308 was identified by peptide mass mapping (Fig. 2*B*).

TABLE II
N-terminal sequences of m-bik and m-pTGF-β

Sequences were obtained by Edman degradation on excised protein bands from SDS-polyacrylamide gels under reducing conditions. *X*, unidentified residue; underlined residues, uncertain assignment.

Gel band molecular mass	N-terminal sequence	Polypeptide
kDa		
15	VTKKEDS(S/C)QL	m-bik
10	ARNGD(D/H)XPLG	m-pTGF-β

Mass Spectrometric Analysis of Urinary Pregnancy hCG Preparations—The anti-HIV-KS active clinical grade urinary hCG preparations CG-10 (Sigma), Pregnyl (Organon), and Profasi (Serono) were subjected to MALDI-TOF MS and compared with the MALDI spectra of HAF (Fig. 6). Within the low molecular mass range (*m/z* 6–18,000), all commercial preparations gave a resolved signal pattern of the α-subunit of hCG (hCGα) resulting from glycoform heterogeneity. Furthermore doubly charged hCGα signals were also observed (Fig. 6, *A* and *C*). In addition, the Pregnyl preparation exhibited considerable levels of hCGβcf. All preparations contained a signal at approximately *m/z* 11,400. A signal at approximately *m/z* 15,800 was only present in the HAF preparation; however, as it was of low abundance it might have been suppressed in the MALDI spectra of other hCG preparations where more total protein (mainly hCG) was present (Fig. 6, *A–C*). Furthermore an intense signal at approximately *m/z* 6,200 in the HAF preparation was also observed as a major species with Pregnyl and as a low abundance signal with CG-10, but it was not present in Profasi.

DISCUSSION

Proteins associated with pregnancy urine and hCG preparations have been shown to reduce *Candida albicans* pathogenesis, induce apoptosis in KS cells, and inhibit HIV replication (22–26). The active protein components have been reported previously to be an 18-kDa RNase closely homologous to eosinophil-derived neurotoxin (22, 23), a 14-kDa hu-

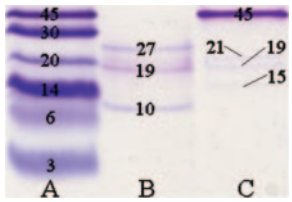

FIG. 5. **Carbohydrate detection in the HAF preparation.** Carbohydrate detection was performed after SDS-PAGE of HAF under non-reducing conditions. *Magenta* bands are positive for sugar moieties. *A*, markers; *B*, HAF preparation; *C*, horseradish peroxidase.

man lysozyme C (24), an RNase A (23 kDa), and a urinary RNase U (~18 kDa) (24). In some cases it has simply been described as a protein complex named HAF that was composed of 15–30- and 2–4-kDa polypeptides (25, 26). The urinary hCG preparation CG-10 (Sigma) is known to be rich in these hCG-associated bioactive polypeptides. The aim of this study was to use modern mass spectrometry to identify these urinary hCG-associated proteins that contaminate pharmaceutical preparations of hCG known to have anti-HIV-KS activity (25, 26).

Tryptic mass mapping by MALDI-TOF MS, PSD MALDI-TOF MS, nESI-MS, and N-terminal sequencing of isolated HAF identified two polypeptides to be fragments of pTGF-β (m-pTGF-β, 11.4 kDa) and bikunin (m-bik, 15.8 kDa) (Figs. 1, 3, 4, and 6 and Tables I and II). There was no evidence for the presence of RNase A, RNase U, eosinophil-derived neurotoxin, or lysozyme C.

pTGF-β is also known as macrophage inhibitory cytokine-1 (MIC-1), growth differentiation factor-15, placental bone morphogenic protein, prostate differentiation factor, and neuregulin-1. It has a mature intact polypeptide mass of 12.5 kDa (Swiss-Prot entry number Q99988, www.expasy.ch) and, by homology to other TGF-βs, forms a disulfide-linked homodimeric protein (32). pTGF-β first appeared in the literature as MIC-1, an autocrine regulatory molecule of macrophage cells (31). It was later shown that the human placenta produced MIC-1 with elevated levels found in maternal serum (33). Antitumorigenic activity of pTGF-β was first demonstrated by Li *et al*. (34) who found that its overexpression could induce cell cycle arrest and apoptosis in breast tumor cells *in vitro*. Subsequent studies showed that its down-regulation could decrease cell cycle arrest and apoptosis in human mammary carcinoma cells (35) and increase the incidence of benign prostatic hyperplasia (36). *In vitro* experiments on cancer cells showed that pTGF-β could reduce cell adhesion followed by cell detachment and induce apoptosis (37, 38). However, some studies have proposed a positive relationship between pTGF-β expression and tumor progression in colorectal carcinoma (39) as well as gastric tumor tissues (40). This is consistent with the bifunctional role of TGF-β, which induces mesothelial cell growth while inducing epithelial cell apoptosis (6).

It is important to bear in mind that what has been detected in these urinary hCG preparations is a fragment of the pTGF-β monomer not the mature protein. Thus any anti-HIV-KS activity is not likely to be via receptor activation but most probably via antagonist mechanisms. Interestingly this is consistent with a previous suggestion that hCGβ fragments blockade the TGF-β receptor complex by virtue of their cysteine knot growth factor structural homology with TGF-β (6). Obviously if this were the case, high levels of a monomeric fragment of pTGF-β would have greater antagonistic properties. However, it is highly probable that the anti-HIV-KS activity of these preparations is not solely due to m-pTGF-β. Clinical studies have clearly shown that effective anti-HIV-KS treatment is only achievable when an antiviral replication agent is used in combination with a protease inhibitor (41).

Bikunin is a proteinase inhibitor containing two Kunitz domains (30). It has various synonyms: inter-α-trypsin inhibitor light chain, urinary trypsin inhibitor, acid-stable trypsin inhibitor, mingrin, urinastatin, and ulinastatin. It is initially expressed as the α_1-microglobulin/bikunin precursor, which is post-translationally modified to contain asparagine (*N*-)linked glycosylation moieties and a very large chondroitin chain. The mass of the unmodified amino acid chain is 16.0 kDa (Swiss-Prot entry number P02760, www.expasy.ch), whereas its intact mass is 25–26 kDa depending on the structure of the post-translational chondroitin and *N*-linked glycosylation chains (30). Aside from its established proteinase inhibitor activity, bikunin also functions as a growth factor in fibroblasts and as an inflammatory acute phase marker protein (30). One study has shown that certain recombinant forms of bikunin can have an inhibitory effect on HIV-1 infectivity *in* vitro (42). In cancers, its expression by human glioblastoma cells can cause suppression of tumor invasion (43), whereas addition of bikunin to human chondrosarcoma cell cultures blocks cell spreading (44).

Although we only detected a fragment of bikunin (m-bik) in these preparations, it is very likely that what remains are active protein Kunitz inhibitor domains. The large m-bik 15.8-kDa fragment contains both Kunitz domains of bikunin, and domain I is the only domain to contain an *N*-linked carbohydrate structure (amino acid position 250 on the precursor molecule) (30). An *O*-linked chondroitin sulfate chain, consisting of disaccharide-repeating units, is attached on the N-terminal extension of bikunin but is not found within m-bik (amino acid position 215 of the precursor molecule) (30). In the present study, m-bik was shown to be glycosylated. In contrast, although pTGF-β contains an *N*-linked glycosylation site at amino acid position 70 of the intact precursor molecule (31), there was no evidence for any carbohydrate structures being present on m-pTGF-β (Fig. 5). This is consistent with the fact that m-pTGF-β was truncated at amino acid 197 on the precursor polypeptide, whereas m-bik was truncated at amino acid 224 on the precursor polypeptide; therefore m-bik retained a possibly metabolized *N*-linked glycosylation at amino acid position 250 (Fig. 2*A*). Bikunin carbohydrates play

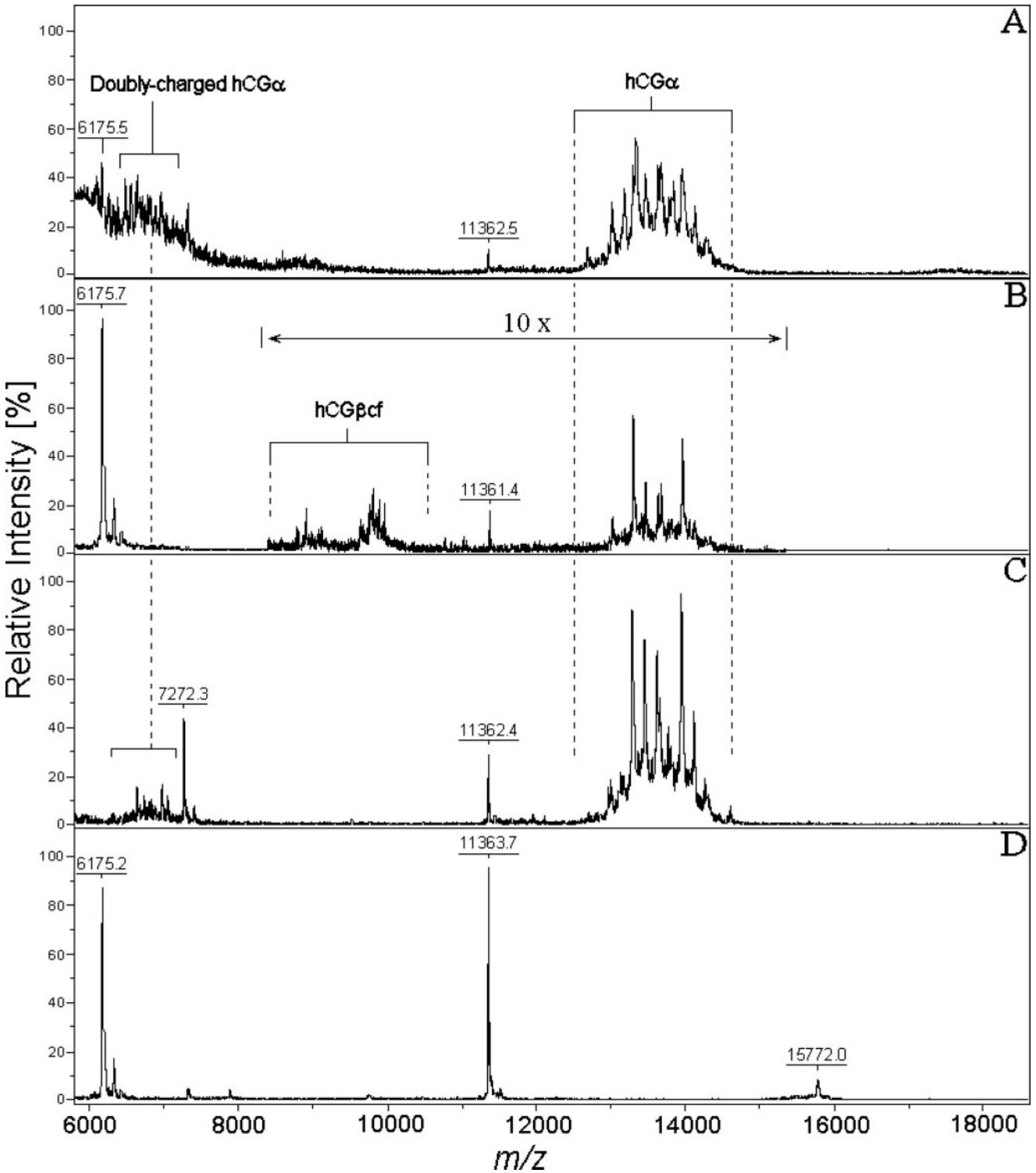

FIG. 6. **MALDI-TOF MS of different hCG preparations.** *A*, CG-10 (Sigma); *B*, Pregnyl (Organon); *C*, Profasi (Serono); *D*, HAF preparation. The spectra focus on the mass region between 6 and 18 kDa. hCGβ was observed in *A*, *B*, and *C* but is omitted here for clarity. The mass region from 8.5 to 15 kDa in *B* is expanded 10×.

a central role in determining its functions, and it is thus possible that m-bik retains its Kunitz domain protease inhibitory properties (45).

It is also significant that MALDI-TOF MS analysis of HAF (Fig. 6*D*) revealed the presence of a 6.2-kDa trypsin resistant component that was not visualized on the gels. It is possible

that, due to its mass resemblance and loss after extensive denaturation during SDS-PAGE, this might have been a further degradation fragment of bikunin consisting solely of Kunitz domain II. A theoretical mass for a bikunin Kunitz domain II fragment, between amino acids 285 and 340 of its precursor protein, is 6,176 Da and therefore matches the unknown peptide detected to within 1 Da of mass accuracy. An alternative candidate trypsin inhibitor exists in the literature. A 6.2-kDa polypeptide protein with antiprotease activity was isolated from the urine of a patient with ovarian cancer (46). It was termed tumor-associated trypsin inhibitor (TATI) but is also known as pancreatic secretory trypsin inhibitor (47). TATI has an intact mass of 6,247 Da (Swiss-Prot entry number P00995, www.expasy.ch), and thus the polypeptide observed at *m/z* 6,175 in this study (Fig. 6*D*) could be a metabolic product of TATI. Its expression has also been noted in pregnancy where TATI is produced at levels similar to those in cancerous conditions (48).

Clearly this study is in conflict with previous data, which propose RNase and lysozyme molecules as the active anti-HIV-KS contaminant components of pregnancy urine and urinary hCG preparations (22–24). In an editorial Darzynkiewicz (7) pointed out the considerable methodological and theoretical flaws in the previously reported studies of the anti-KS factor found in urinary hCG preparations. Indeed he stated that identification of this anti-KS factor contaminating hCG was essential for further progress in the clinical application of hCG. The proteomic mass spectrometric techniques used in this study are far more discriminatory than immunoblotting and catalytic enzymatic assays used in other reports. Indeed none of the previously proposed molecules were observed in any of the examined hCG preparations.

In 1998 a publication by Lunardi-Iskandar *et al.* (25) stated that identification of the active moiety(ies) of HAF from clinical grade pregnancy urine hCG preparations was one of the high priorities for the initiation of clinical trials where the biological properties of the recognized components could be investigated. We have now identified the anti-HIV-KS proteins closely co-eluting with hCGβcf from the pregnancy urine hCG preparations CG-10 (Sigma), Pregnyl (Organon), and Profasi (Serono) as m-pTGF-β, m-bik, and possibly a metabolic product of TATI. In general terms, this concurs with the original studies of Lunardi-Iskandar *et al.* (25) in which they proposed HAF to be composed of several polypeptides ranging from 2–30 kDa. Now that we have identified the contaminant proteins in commercial hCG preparations with anti-HIV-KS activity, the possible role of hCG in HIV-KS treatment can be thoroughly investigated.

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