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Peptides from the Bursa of Fabricius Associated with Suppression of Mitogen Stimulated DNA - Synthesis in Bursa of Fabricius Cells Belong to Intracellular Proteins

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Abstract: Previous reports from our laboratory have demonstrated that an reverse phase HPLC (rpHPLC) fraction obtained from extracts of the chicken bursa of Fabricius posses both *in vitro* anti-steroidogenic activity on avian and mammalian cells and suppression on mitogen stimulated DNA-synthesis in chicken BF cells. Utilizing YM cut-off membranes the bioactive fraction appears to be between ~3-5kDa. However, the identity of such peptide (s) remains unknown. Here, subjected those peptides for mass spectrometric (MS) nano-electrospray quadruple time-of-flight (Q-TOF) MS/MS analysis in an effort to elucidate the composition of predominant fragments. The results of these analyses indicate the presence of small fragments of the non-histone chromosomal protein high mobility group (HMG), nucleophosmin, elongation factor 1-alpha, thymosin β4 (Tβ4), thymosin β, stathmin and histone H1.10. These results indicate that the suppression present in the rpHPLC fraction obtained from the BF, rather than been recognized extracellular messengers, like hormones or cytokines, contains intracellular molecules.

Key words: Intracellular, anti-proliferative, anti-steroidogenic and bursa of Fabricius

Introduction

The bursa of fabricius (BF) is a primary immune organ in avian species where early B lymphocyte proliferation and differentiation take place (Cooper *et al*., 1966; Glick *et al*., 1956; Lydyard *et al*., 1976). In addition, the BF has been postulated to play a role in the immune-endocrine axis by producing a putative anti-steroidogenic factor (Glick, 1984; Romano *et al*., 1981; Pedernera *et al*., 1985; King *et al*., 1985), which identity remains elusive. Caldwell *et al*., 1998 and 1999 found that a protein of ~32kDa extracted and purified from the BF with remarkable anti-steroidogenic and anti-proliferative activity on both avian and mammalian cell culture assays, which was first thought to be a hormone, has undistinguishable relationship with the chicken histone H1 family proteins (Garcia-Espinosa *et al*., 2002). Controversially, a rpHPLC and YM cut-off membranes purification, containing heat-labile, basic and blocked at both the amino and carboxyl termini peptides extracted from the BF with an apparently molecular weight of ~3-5kDa, shown similar suppression bioactivities on avian and mammalian cell culture assays (Byrd *et al*., 1993; Byrd *et al*., 1994; Byrd *et al*., 1995).

Due the unknown identity of these peptides it is not possible to establish whether or not they belong to HH1 or they are novel peptides. Therefore, our objective was to subject those peptides to mass spectrometric (MS) nano-electrospray quadruple time-of-flight (Q-TOF) MS/MS analysis to know their identity.

Materials and Methods

Extraction and purification of the bioactive peptides: The anti-steroidogenic and anti-proliferative peptides were extracted and purified following our previous published protocol (Byrd *et al*., 1993). Instead of utilized the 5kDa YM-cut off membrane we used a 10kDa FROM Millipore (Bedford, MA USA). Briefly, BF were collected from 7-week-old chickens immediately after death and held at -76°C prior to extraction. Tissue (200g) was homogenized with 2 parts 15% trifluroacetic acid (TFA) (Sigma, St. Louis, MO. USA). Homogenized material was centrifuged twice at 37,000 x g for 20 minutes at 4°C. The resulting supernatant was then carefully removed, avoiding contamination with surface lipid and loaded onto 5mM TFA-equilibrated 2.5 x 10cm preparative C-18 Mega bond elut columns (Varian, Harbor City, CA, USA). Cartridges were stepwise eluted (15 ml) with 0, 20, 60 and 80% acetonitrile (ACN) with 5 mM TFA (v:v) and eluent was collected in polypropylene tubes and dried by vacuum centrifugation. The 60% ACN fraction, which previously was shown to contain the bioactive peptides (Byrd *et al*., 1993) was reconstituted in double distilled water and filtered (0.2µm) prior to concentration and dialysis. The solution was concentrated and dialyzed sequentially against YM-30, YM-10 and YM-3 kDa centriplus units, adding milli-Q water to the sample 10 times and centrifuged at 2000 x g for 45 minutes (Amicon, Bedford, MA, USA). Resulting samples were dried by vacuum centrifugation, reconstitute in one ml of

Table 1: Amino acid sequences obtained from the mass spectrometric analysis on chicken (Gallus gallus) BASP bioactive protein/peptides of 3-10 kDa were alignment against (Gallus gallus) GenBank databases. Some fragments match quail (Coturnix coturnix japonica) thymosin ß [§]. Coverage of fragment sequences is shown in boldface and acetylated residue in *.

Peptide/protein	MW (kDa)	# AA	Sequence (coverage)			# tryptic fragments found	Coverage (%)	Acc no.
Thymosin β4	4.9	43	*SDKPDMAEIE ETQEKNPLPS	 KETIEQEKQA	KFDKSKLKKT GES	9	90.7	gi:27591540
§Thymosin β	5.4	44	*CDKPDLSEVE NTEEKNTLPS	 KETIEQEKEC	KFDKKKLKKT VKSS	9	88.6	gi:11342542
High mobility group type 17	9.2	90	MPKRKAEGDT PQRRSARLSA PKKAAPKKSE DAGKEGNNPA QKAEGAGDAK		KGDKAKVKDE KPAPPKPEPK KVPKGKKGKA ENGDAKTDQA	12	86.6	gi:123105
High mobility group type 14a	10.4	105	MPKRKAPAEG ARLSAKPAPP PKKEKAANDK KKGAKGKDET SENGDTKTNE	 APAAEASDDK	EAKEEPKRRS KPEPKPKKAA KEDKKAATKG KQEDAKEENH EAKSE	6	48.6	gi:123103
Stathmin	19.0	161	MATSDIQVKE AAPEFPLSPP RKSHEAEVLK NNFSKMAEEK NREAQMAAKL EEVRKNKEGK	LEKRASGQAF KKKDLSLEEI QLAEKREHEK DPGEAETN	ELILGPRSKE QKKLEAAEER EVLQKAIEEN LTHKMEANKE ERLREKDKHI	4	27.7	gi:63797
Elongation factor 1-alpha	50.0	462	MGKEKTHINI CGGIDKRTIE KGSFKYAWVL ETSKYYVTII CAVLIVAAGV YTLGVKQLIV VKEVSTYIKK MLEPSSNMPW GNASGTTLLE QDVYKIGGIG VLKPGMVVTF SVEMHHEALS NVKNVSVKDV NDPPMEAAGF LDCHTAHIAC PKFLKSGDAA LGRFAVRDMR VDKKAGGAGK	VVIGHVDSGK DKLKAERERG DAPGHRDFIK GEFEAGISKN GVNKMDSTEP IGYNPDTVAF ALDCILPPTR TAQVIILNHP KFAELKEKID IVDMIPGKPM VTKSAQKAQK	STTTGHLIYK KFEKEAAEMG ITIDISLWKF NMITGTSQAD GQTREHALLA PYSQKRYEEI VPISGWNGDN FKGWKVTRKD PTDKPLRLPL TVPVGRVETG APVNVTTEVK EALPGDNVGF RRGNVAGDSK GQISAGYAPV RRSGKKLEDG CVESFSDYPP QTVAVGVIKA AK	4	10.8	gi:3122072
Nucleophosmin	32.6	294	MEDSAMDMES FGCELKAEKE EHQLSLRTVT GNPTKVVLAS VLRLKCGSGP LEEEPESEDE TKRPASGGGA EDDEDDDEDE DDDEEEIKTP KNMQKAKQNG KTKTPDSKKD MQASVDKGCS YVKNCFRTED	 LGAGAKDELH LKMSVQPTVS KSLTPKTPKV QKVIQALWQW	MGPLRPQTFL YQFKVDDEEN VVEAEALDYE LGGFEITPPF VYVSGQHLVA EEDTKIGNAS KTPQKKPKLS DDDEDDEDDL MKKPAREPAG KDSKPSTPAS PLSLEEIKAK LPKLEPKFAN RQTL	3	9.2	gi:128413
Histone H1.10	21.8	219	*SETAPAAAPA KKPKKAAGGA TELITKAVSA YDVEKNNSRI ASGSFRLSKK KRTPAAKPKK KKPKKAAAAK PKKATKAAKP KPKAAKPKAA	 SKERKGLSLA KLGLKSLVSK KSPKKAKKPA KKAATAKSPA KAKKAAAKK	VAAPAAKAAA KARKPAGPSV ALKKALAAGG GTLVQTKGTG PGEVKEKAPR PAAKKPASAA AAATKKAAKS KAKAVKPKAA	3	16.9	gi:2506498

Milli-Q water and stored at -20°C until use. The bioactivity of the peptides was corroborated with mitogen-stimulated DNA-synthesis BF cells assay (data not shown).

Mass spectrometric analysis: Samples were digested by trypsin and analyzed by nanoflow HPLC (Ultimate, LC-Packings, Amsterdam, The Netherlands) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK). One hundred µl of the sample was SpeedVac concentrated to 10 µl, reduced with DTT and alkylated with iodoacetamide. Tryptic digestion was done overnight at 37°C, in a 40 µl solution containing 25 mM NH_4 HCO_3, 0.1 µg modified porcine trypsin (Promega, Leiden, The Netherlands) and 2% (v:v) ACN.

The nanoflow HPLC was run using the following conditions: 10 µl of the digest was loaded on the precolumn (using an isocratic flow of 2% ACN, 0.1% formic acid (FA), pumped at 10 µl/min) and reverse-phase eluted over the capillary PepMap column at a flow rate of 150 nl/min, using a linear gradient of 95% A, 5% B to 35% A, 65% B in 38 min, followed by a linear gradient to 5% A, 95% B in 2.5 min and then to 95% A, 5% B in 2.5 min and held for 2 min (solvent A: 0.1% FA in HPLC-grade water; solvent B: 0.1% FA in ACN). The column outlet was coupled to the Q-TOF mass spectrometer through a SilicaTip capillary (New Objective Inc., Woburn, MA, USA) at 1200-1700 V, with the cone voltage at 25 V. In the mass spectrometer, doubly, triply and quadruply charged peptides were automatically selected and fragmented using predefined collision energy profiles, depending on the detected peptide charge. The procedure was repeated three times, with stepped, narrow m/z ranges to which peptide detection was limited (400-600, 600-800, 800-1200 m/z) to ensure fragmentation of ions of lower abundance.

The data files were processed with the ProteinLynx software (Micromass) to produce peak list files (*PKL). These 3 files were concatenated and submitted to the Mascot search engine (Matrix Science, London, UK), using the MSDB database.

Results and Discussion

Mass spectrometric Q-TOF MS/MS analysis of the peptides between the range of ~3 to 10 kDa, capable of preventing DNA-synthesis, displayed several fragments with a 100% match to seven different known chicken peptides/proteins and one to quail thymosin beta (Table 1). The amino acid alignment shows thymosin β4, thymosin β and non-histone chromosomal protein HMG-17 with the highest amino acid coverage of all sequences (>80%). Non-histone chromosomal protein HMG-14A shows some amino acid coverage (48%), while stathmin, elongation factor 1-alpha, nucleophosmin and histone H1.10 covered less than 30%. The quest to identify the anti-steroidogenic molecule from the BF has recently pointed to the histone H1 family (Garcia-Espinosa *et al.*, 2002). However, small anti-steroidogenic peptides placed to be between ~3 to 5 kDa (Byrd *et al.*, 1993) purified from the BF and subjected to mass spectrometric Q-TOF MS/MS analysis have indicated that those peptides sequences are different to the HH1 family proteins. Our results clearly demonstrate that most of the peptide sequences found belong to either nuclear or cytoplasmic proteins, which are above 4.9 kDa (Table 1). The molecule responsible for the suppression bioactivity is unknown, but some peptides present in our fraction might represent attractive possibilities. For instance, thymosin β4 (4963 Da) with either natural or oxidative modification has shown multiple bioactivities (Huff *et al.*, 2001). Particularly interesting is the N-terminal fragment (SDKP) of thymosin β4, which is associated with inhibition of pluripotent stem cell proliferation (Lenfant *et al.*, 1989). Further, the 21 amino acid peptide from the C-terminal region (69-89) of the non-histone chromosomal protein HMG-17 has been shown to suppress tumor invasion (Isoai *et al.*, 1992). In addition, it is known that nuclear peptides or proteins can act like extracellular signal molecules (Brown *et al.*, 1997).

These results clearly indicate a difference between the ~3-5 kDa peptides (Byrd *et al.*, 1993) and the ~32 kDa protein (Caldwell *et al.*, 1999) which is now believed to be HH1 (Garcia-Espinosa *et al.*, 2002). Further peptide characterization is needed to identify which peptide or peptides have the most potent bioactivity and whether or not there are multiple bioactivities associated with one specific peptide.

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