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Short communication

Identification of T cell receptor signaling pathway proteins in a feline large granular lymphoma cell line by liquid chromatography tandem mass spectrometry

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ABSTRACT

Tryptic peptides of a feline large granular lymphoma cell line were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS). Seventeen proteins of the T cell receptor signaling pathway could be identified by this approach. So far the existence of these proteins has only been postulated in the protein databases while experimental proof of their expression is predominantly pending. This article suggests where these proteins are located within the T cell receptor signaling pathway, thereby giving a short overview of the structure and function of this cascade.

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1. Introduction

The accessible publication databases do not contain any comprehensive investigation of the feline T cell receptor (TCR) signaling cascade. The human T cell receptor signaling pathway is known in detail (recently reviewed by Brownlie and Zamoyska, 2013) and a model of the feline signaling cascade is provided by the "Kyoto Encyclopedia of Genes and Genomes" (KEEG) deduced from public sequence database data (http://www.genome.jp/kegg/).

With the sequencing and comparative analysis of the feline genome (Pontius et al., 2007) sequence information of many feline genes has become available. Due to automatic annotation (Curwen et al., 2004) gene function, transcript information, and predicted protein sequences for many genes are available in public databases, e.g. Universal Protein Resource (UniProt). However, these sequences, especially the translation products, should be considered

Abbreviations: ADAP, adhesion- and degranulation-promoting adaptor protein; CBL, E3 ubiquitin-protein ligase CBL (casitas B-lineage lymphoma proto-oncogene); CD, cluster of differentiation; CDC42, cell division control protein 42 homolog; DTT, dithiothreitol; GRAP2, GRB2-related adaptor protein 2; GRB2, growth factor receptor-bound protein 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAA, iodoacetamide; ITK, interleukin-2-inducible T cell kinase; KEEG, Kyoto Encyclopedia of Genes and Genomes; LAT, linker for activation of T cells; LC–(ESI)–MS/MS, liquid chromatography–(electrospray ionization) tandem mass spectrometry; LCK, lymphocyte-specific protein tyrosine kinase; NCK1, non-catalytic region of tyrosine kinase adaptor protein 1; NFAT, nuclear factor of activated T cells; PAK, p21 protein (Cdc42/Rac)-activated kinase; PLCγ1, phospholipase Cγ1; SHP1, SH2 domain-containing phosphatase 1; SLP76, SH2 domain-containing leukocyte protein of 76 kDa; TCR, T cell receptor; UniProt, Universal Protein Resource; VAV1, proto-oncogene vav; ZAP70, zeta-chain associated protein kinase of 70 kDa.

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as preliminary data until experimental proof of expression in vivo can be given.

The feline T cell receptor repertoire itself has been characterized experimentally on genomic and transcriptional level (Cho et al., 1998; Cho et al., 1997; Lee and Cho, 1999; Moore et al., 2005; Weiss et al., 2008, 2010, 2012) but experimental proof of the expression of proteins putatively involved in the signaling cascade is sparse with exception of some surface molecules widely used for the recognition and subtyping of T cells (e.g. CD3, CD8, CD4).

During the characterization of a novel feline large granular lymphoma cell line we identified components of the T cell receptor signaling pathway by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS), thereby giving experimental proof of their expression in vivo. The cell line itself showed morphological features of large granular lymphocytes, and a rearrangement of T cell receptor gamma genes but not of B cell receptor genes could be identified by PCR (according to Moore et al., 2005 and Henrich et al., 2009; data not shown). A detailed characterization of the cell line will be subject of a further publication.

2. Materials and methods

2.1. Cell homogenization

Four aliquots of the feline large granular lymphocyte cell line “S87” (10^7 cells/ml) were centrifuged with $120 \times g$ for 10 min and washed twice in precooled (4 °C) lysis buffer (255 mM sucrose [Carl Roth, Karlsruhe, Germany], 20 mM HEPES [Carl Roth, Karlsruhe, Germany], 1 M Titriplex(III) [Carl Roth, Karlsruhe, Germany], pH 7.4). Cells were mechanically homogenized using a Bullet blender® (Next Advance, Averill Park, New York, USA) according to the manufacturer’s instructions. Shortly, the supernatant of the pellet was removed and zirconium oxide beads (0.15 mm, Next Advance, Averill Park, New York, USA) in volume equal to the volume of the cell pellet was added. A volume of buffer twice the volume of the pellet was added thereafter and the cells were lysed 3 min with the Bullet blender®. The supernatant was transferred into a new Eppendorf tube and placed on ice.

2.2. Sample preparation for liquid chromatography–electrospray ionization tandem mass spectrometry

The samples were treated with 5 mM dithiothreitol (DTT, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (57 °C for 45 min) in order to reduce disulfide bounds and alkylated with 15 mM iodoazetamide (IAA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (at room temperature for 45 min in the dark). Protein digestion was achieved by use of modified trypsin (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, Wisconsin USA) at a 1:30 enzyme/protein (w/w) ratio at 37 °C for 15 h. An additional purification step using C18 Zip Tips (Varian, Lake Forest, California USA) was applied according to the manufacturer’s recommendations.

2.3. Liquid chromatography–electrospray ionization tandem mass spectrometry

Chromatographic separation was performed on an Ultimate binary nano-high-performance liquid chromatography (nano-HPLC) pump/autosampler system (LCPackings/Dionex, Idstein, Germany). Volumes of 1 μL of the sample were pre-focused on a trap column (Dionex, C18 PepMap, i.d. 300 μm, length 5 mm) and afterwards separated on a fused-silica Acclaim PepMap 100 C18 capillary column (Dionex) with 3 μm particle size, 100 Å pore size, 75 μm inner diameter and 15 cm length. The flow rate was set to 0.3 μL/min. Solvent A consisted of water with 2% acetonitrile (Merck Millipore, Darmstadt, Germany) and 0.1% formic acid (v/v) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and gradient elution solvent B consisted of acetonitrile with 20% water and 0.1% formic acid (FA) (v/v). Separation was performed as follows: Pre-concentration lasted for the first 3 min of the HPLC run with 0% B. The solvent B was increased to 6% within 2 min and afterwards to 45% in 85 min. For cleaning the column solvent B was further increased to 95% within 10 min and maintained for 10 min. Then the gradient was ramped down to 0% solvent B within 2 min. The nano-HPLC was attached to a nano-electrospray interface of a Q Exactive mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). All measurements were done in positive ion mode and a signal of a common polysiloxane m/z 445.12003 was used as a lock mass for internal calibration. In full scan mode the value of the automated gain control (AGC) target was set to 1E6 and the maximum injection time was 60 ms. The ten most intense peaks from full scan with a mass resolution of $R = 70{,}000$ (@m/z 200) were used for fragmentation. Unassigned and singly charged ion peaks were excluded. Higher-energy collision dissociation (HCD) was used with normalized collision energy of 28% for fragmentation. The isolation window for the precursor was set to 2.0 Da. The mass resolution for MS/MS was set to $R = 17{,}500$ (@m/z 200). Each of the four samples was measured three times and the data of the 12 runs were combined for data analysis.

2.4. Database search

The LC–ESI–MS/MS data were processed with Proteome Discoverer software version 1.4 (Thermo Fisher Scientific GmbH, Bremen, Germany) and searched against the UniProtKB database (accessed on 1 July 2013) filtered with *Felis catus* taxonomy based on SEQUEST search algorithm. The mass tolerance for precursor ions was set to 5 ppm and the fragment ion mass tolerance was 0.02 Da. The search was performed choosing trypsin as enzyme and up to two missed cleavages were allowed in order to account for incomplete digestion. Carbamidomethylated cysteine (+57.021) was used as a static modification and oxidation (+15.995) was set as a dynamic modification. Peptide validation was done with the Percolator algorithm. Filtering was performed with “high confidence” which correspond to a q-value <0.01 and a false discovery rate (FDR) of 1%, as determined by a decoy database search. Proteins were considered as identified if at least two corresponding peptides were detected.

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