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Use of affinity-directed liquid chromatography-mass spectrometry to map the epitopes of a factor VIII inhibitor antibody fraction

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Summary

Background—Neutralizing factor (F) VIII antibodies develop in ~30% of individuals with hemophilia A and show specificity to multiple sites in the FVIII protein.

Methods—Reactive epitopes to an immobilized IgG fraction prepared from a high-titer, FVIII inhibitor plasma were determined following immuno-precipitation (IP) of tryptic and chymotryptic peptides derived from digests of the A1 and A2 subunits of FVIIIa and FVIII light chain. Peptides were detected and identified using highly sensitive liquid chromatography-mass spectrometry (LC-MS).

Results—Coverage maps of the A1 subunit, A2 subunit and light chain represented 79%, 69% and 90%, respectively, of the protein sequences. Dot blots indicated that the inhibitor IgG reacted with epitopes contained within each subunit of FVIIIa. IP coupled with LC-MS identified 19 peptides representing epitopes from all FVIII A and C domains. The majority of peptides (10) were derived from the A2 domain. Three peptides mapped to the C2 domain, while two mapped to the A1 and A3 domains, and single peptides mapped to the a1 segment and C1 domain. Epitopes were typically defined by peptide sequences of <12 residues.

Conclusions—IP coupled with LC-MS identified extensive antibody reactivity at high resolution over the entire functional FVIII molecule and yielded sequence lengths of less than 15 residues. A number of the peptides identified mapped to known sequences involved in functionally important protein-protein and protein-membrane interactions.

Keywords

factor VIII; inhibitor plasma; epitope mapping; mass spectrometry

Introduction

Neutralizing anti-factor VIII antibodies develop in approximately 30% of individuals with hemophilia A and represent a significant complication in treating this disease with replacement factor VIII (FVIII) therapy (see Refs [1, 2] for review). FVIII circulates as a procofactor that requires proteolytic activation to convert FVIII to the active cofactor FVIIIa. Once activated, FVIIIa binds FIXa in a membrane-dependent interaction to form the FXase complex, responsible for the efficient conversion of FX to Xa during the propagation

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phase of coagulation (see Ref [3] for review). Neutralizing antibodies or inhibitors have been shown to block multiple functions in this process including procofactor activation, complex formation, and substrate catalysis.

The domain sequence of FVIII is represented as: A1(a1)A2(a2)B(a3)A3C1C2, with the lower case *a* representing short segments rich in acidic residues [4]. The primary circulating form of FVIII is a non-covalent heterodimer comprised of a heavy chain (A1(a1)A2(a2)B domains) and a light chain ((a3)A3C1C2 domains). Activation of FVIII yields the FVIIIa heterotrimer of A1, A2 and A3C1C2 subunits resulting from cleavage at the a1-A2, a2-B and a3-A3 boundaries. Several protein-protein interactions critical to formation and function of FXase have been mapped to the A2 domain, whereas the C2 domain provides interactive sites for membrane binding. These two structures appear to represent primary sites for binding of inhibitory antibodies.

A number of techniques have been employed to detect and/or map inhibitor antibody binding sites. These methods have focused on immuno-precipitation (IP) and/or Western blotting of FVIII fragments, either derived from the FVIII protein [5], expressed in *E. coli* [6], as phage displayed libraries [7] or as synthetic peptide arrays [8]. In addition, results using human-porcine chimeras have identified inhibitor epitopes within A2 [9], C2 [10] and a3 [11]. In an earlier report [12], we mapped the epitope for the monoclonal antibody, R8B12, to a discontinuous epitope within the A2 domain using an affinity-directed matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) technique. We now use an affinity-directed method employing the more robust and sensitive liquid chromatography (LC)-MS for detection of epitopes reactive with an inhibitor plasma IgG fraction that recognizes multiple domains in FVIII. Results from this analysis identify 19 peptides representing antibody epitopes from all FVIII A and C domains, with the majority of peptides derived from the A2 domain. In most cases, epitopes are defined by peptide sequences of <12 residues. A number of these peptides mapped to known sequences important for protein-protein and protein-membrane interactions.

Materials and Methods

Reagents

FVIII inhibitor patient plasma (533 BU, lot GK 1838-1156) was obtained from George King Bio-Medical (Overland Park, KS). Recombinant FVIII (Kogenate™; Bayer Corp., Berkeley, CA) was a gift from Dr. Lisa Regan. The monoclonal FVIII A2 antibody R8B12 was obtained from Green Mountain Antibodies (Burlington, VT). MS grade trypsin was purchased from Promega (Madison, WI) and epoxy-activated agarose and chymotrypsin were purchased from Sigma (St Louis, MO).

Isolation of FVIII Light Chain and A1 and A2 Subunits and Proteolytic Cleavage

FVIII heavy and light chains were isolated as previously described [13]. The heavy chain was treated with thrombin and resultant A1 and A2 subunits were separately purified as described [13]. Subunits were >95% pure as judged by SDS-PAGE. Protein was dialyzed into 50 mM ammonium bicarbonate, pH 8, reduced, alkylated, and digested with either trypsin or chymotrypsin (25:1 wt/wt) overnight at 37 °C. Samples were reacted with an additional aliquot of protease for 6 h before terminating the reaction with TFA (0.1%) final concentration.

Isolation of IgG from Plasma

FVIII inhibitor patient plasma (3 mL) was treated with EDTA (10 mM final concentration), and precipitated with ammonium sulfate (45%) at 4 °C. Following centrifugation (4000 x g),

the pellet was resuspended in 0.5 ml of PBS and dialyzed into PBS overnight. The protein was loaded onto a HiTrap Protein G (GE Healthcare, Uppsala, Sweden) column equilibrated in 20 mM HEPES pH 7.2, 0.15 M NaCl, 0.02% NaN₃, and eluted with 0.1 M glycine pH 2.5. Fractions (1 mL) were collected and neutralized with 400 μ L 1 M Tris HCl pH 7.5. Peak fractions were pooled and dialyzed into Coupling Buffer (100 mM Na₂CO₃ pH 9.5, 100 mM NaCl).

Dot Blots

Purified FVIIIa A1, A2, A3C1C2 subunits (0.1-1 μ g) were spotted onto PVDF, the paper blocked and then reacted using the purified IgG fraction (6 μ g/mL) from the inhibitor plasma. Alkaline phosphatase-conjugated goat anti-human IgG was used as the detection antibody.

Preparation of IgG-Coupled Epoxy-Activated Agarose

Epoxy-activated agarose (EAA) resin (~0.5 g) was swollen in water, rinsed several times in Coupling Buffer, and incubated overnight at 37 °C with ~5 mg IgG. The suspension was centrifuged and the resin was resuspended in several volumes 1 M ethanolamine pH 8, and incubated 4 hours at 4 °C with mixing. Beads were then washed two times each with PBS; 0.1 M sodium acetate pH 4.0, 0.5 M NaCl; 0.1 M sodium bicarbonate pH 8.0, 0.5 M NaCl; and again with PBS before storage at 4 °C.

Immunoprecipitation

FVIII peptides from the light chain digest or combined from the A1 and A2 subunit digest (3-fold molar excess over bound IgG) were added to the IgG-coupled EAA resin (65 μ L) in a 1 mL Eppendorf LoBind tube, 50 mM ammonium bicarbonate (500 μ L) was added and the mixture was incubated 1 hour at 4 °C with mixing. The beads were then washed three times by centrifugation with PBS, and then once with 1 mL 50 mM ammonium bicarbonate. The washed beads were treated with 40 μ L of a volatile acid (500 mM acetic acid, 20 mM ammonium acetate, pH 3.5) to elute the bound peptides, which were transferred to a LoBind tube containing 120 μ L of 50 mM ammonium bicarbonate to immediately neutralize the sample. A total of three acid treatments were combined, and samples were lyophilized and resuspended in 10 μ L of 50 mM ammonium bicarbonate.

Mass Spectrometry Analysis of Peptide Epitopes

A C18 reverse phase nanospray tip was loaded with ~20% of the peptide digest and washed with 5% methanol, 0.1% formic acid, for 10 min. Peptides were eluted and analyzed by LC-MS/MS using a Thermo LTQ mass spectrometer and the following sequential solvent steps: 5-15 % methanol gradient (2.5 minutes), 15-60% methanol gradient (67 minutes), 60% methanol (4 minutes), and 95% methanol (3 minutes), with all solvents containing 0.1% formic acid. A full MS survey scan was performed every 3 seconds and the top 7 peaks were selected to produce MS/MS fragmentation spectrum. Following the LC-MS/MS run, the full MS spectra and the MS/MS fragmentation data were used initially in a Mascot search (www.matrixscience.com [14]) of the entire human proteome, and subsequently in custom proteomes containing FVIII sequences. Search criteria used for selecting fragmentation spectra that map to proteolytic peptides were: peptide tolerance = -0.8 to $+0.5$, a minimum ion score of 15, and fragmentation spectrum with expect values significantly lower than the score value. One missed cleavage was tolerated for trypsin-treated samples and two to three missed cleavages were allowed for chymotrypsin-treated samples.

Results

Sequence Coverage of FVIII Light Chain and the A1 and A2 Subunits of FVIIIa

Sequence coverage maps were generated using the purified A1 (residues 1-372) and A2 (residues 373-740) subunits of FVIIIa, and the FVIII light chain (residues 1649-2332). The rationale for employing A1 and A2 subunits rather than intact FVIII heavy chain was to eliminate the heavily glycosylated B domain [4] which would yield a complex peptide map difficult to resolve by MS techniques. Furthermore, few if any neutralizing antibodies appear to map within the B domain of FVIII [1].

Tryptic digestion of the isolated A1 and A2 subunits under denaturing conditions produced peptides covering 56% of the heavy chain sequence (residues 1-740), as observed by LC-MS from the separate subunit digests. A chymotryptic digestion of the isolated subunits provided additional peptides that overlapped and complemented the tryptic digestion, giving 74% coverage of this sequence with the two proteases (Fig. 1A). While somewhat greater overall coverage was observed with the A1 subunit (79%) compared with the A2 subunit (69%), digestion of FVIII light chain (a3A3C1C2 domains) using both proteases produced peptides covering 90% of this sequence (Fig. 1B). The greater coverage of light chain compared with the heavy chain sequences likely represent the non-random distribution of trypsin sites in the latter, with several regions of heavy chain having clustered Lys and Arg residues while other regions were largely devoid of these sites. Overall, the two-protease approach resulted in minimal gaps in sequence coverage with significant gaps (>20 residues) identified at residues 29-50 and 214-240 in the A1 subunit, and 595-620 in the A2 subunit. In contrast, only a single gap in coverage of greater than 10 residues in the light chain was observed at N-terminal residues 1649-1672.

Characterization of the Inhibitor IgG Fraction

Total IgG was purified from the plasma obtained from a hemophilia A patient possessing a high titer (533 BU/ml) inhibitor as described in Methods. This inhibitor plasma as well as the IgG fraction purified from the plasma showed complete inhibition of FVIII activity in a clotting assay (results not shown). Dot blots indicated reactivity of the IgG to each FVIIIa subunit (Fig 2), with spot density increasing with increasing amount of FVIIIa subunit. Based upon spot density, it appeared the A3C1C2 subunit possessed the greatest reactivity to the IgG suggesting a relatively high fraction of reactive antibodies and/or antibodies of greater avidity mapped to this subunit. Coupling of the IgG in high concentration (up to 1.2 $\mu\text{g}/\mu\text{L}$) to the EAA beads provided an immuno-affinity reagent for FVIII. The IgG-coupled beads also markedly increased the clotting time of FVIII using a one-stage assay (data not shown). Thus the reactivity of the agarose-bound antibody retained inhibitory activity towards FVIII and did not appear to be appreciably affected by the coupling chemistry.

Immunoprecipitation of Peptides with Agarose-bound IgG

The inhibitor IgG-coupled beads were reacted with peptides derived from protease digests of the FVIII samples. Following extensive washing, peptides that bound to the IgG were eluted, and sequences were identified by LC-MS. LC-MS techniques proved superior to preliminary studies using MALDI-TOF MS, which was used earlier in mapping the epitope for a monoclonal antibody [12]. This result likely reflected the greater sensitivity of the LC-MS to the markedly small fraction of anti-FVIII IgG molecules in the total IgG pool. As a result of this sensitivity, enrichment of IgG for FVIII-specific sequences prior to IP was not required. Control experiments using the beads alone or beads to which was bound an anti-factor VIII monoclonal antibody (R8B12) with known epitope were employed to eliminate any peptides that bound in a non-specific manner.

LC-MS/MS Analysis of Immunoprecipitated Peptides

LC-MS/MS analysis of IP samples from tryptic and chymotryptic digestions of FVIII light chain and FVIIIa A1 and A2 subunits were performed as described in Methods. Results from a typical LC-MS/MS run are shown in Figure 3, which details the analysis in identifying a precipitated peptide in the chymotryptic digest of FVIII light chain. The chromatogram in Figure 3A shows a separation of peptides with the vertical axis representing a cumulative intensity for all peptide ions that elute over each time point. Reverse phase elution profiles of peptides derived from the immunoprecipitations were significantly reduced in signal intensity (data not shown). Subsequent Mascot search analysis of these separations against the human proteome database revealed 185 peptide queries that were derived from the total chymotrypsin digest of the FVIII light chain. Mascot searches were conducted with an ion score threshold cut-off value of 15 in a search that allowed for 1, 2 or 3 missed peptide cleavages, depending on the protease as described in Methods, where the ion score represents a statistical measure of how well the fragmentation spectrum of this ion matches to the spectrum expected for this protein sequence [14].

Peptide mass tolerance was set at 1.5 daltons and peptides were rated as acceptable matches if they had a ± 0.8 delta value (difference between experimental and expected mass calculations). For example, at 18.4 minutes in the reverse phase gradient, a double charged peptide with a mass-to-charge ratio of 629.6 m/z was present in the total digest (Fig. 3B) and also at a similar point in the reverse phase gradient for the immunoaffinity purified peptides (Fig. 3C). MS/MS fragmentation analysis of this peptide reveals b- and y- fragment ions from the peptide sequence: LISSQDGHQW, covering essentially every N- and C-terminal position in the experimental fragmentation spectrum, when the peptide is derived from the total chymotrypsin digest (data not shown). A nearly identical fragmentation spectrum was mapped to the same peptide sequence in the IP sample (Fig. 2D). The combined full and fragmentation spectral data were used to assess the total peptide sequence coverage of the protein.

FVIII peptides identified by IP and LC-MS/MS

Results from IP coupled with LC-MS/MS analysis identified 19 peptides representing antibody epitopes from 16 regions within the factor VIII protein (Table 1). Two of the peptides, designated 9B and 13, were also identified in preliminary experiments using affinity-directed MALDI-TOF MS (results not shown) as previously described [12]. The majority of peptides identified (10) mapped to within the A2 domain, a region known for its high reactivity with inhibitor plasmas [15]. However, little reactivity was observed with A1 domain-derived peptides, with two peptides precipitated representing ~5% of this domain. One peptide was identified as derived from the a1 acidic C-terminal region of the A1 subunit. Of the six peptides precipitated from FVIII light chain-derived sequences, three were derived from the C2 domain, another region known to be highly reactive with inhibitors [15]. A single peptide was identified that mapped to C1 while two peptides mapped to A3 domain sequences. In a control experiment, synthetic peptides prepared to two of the sequences identified (9B and 13, Table 1) were individually subjected to immunoprecipitation and LC-MS using similar conditions as employed for the FVIII digests. Identification of both peptides (results not shown) served to help confirm the specificity of the methods employed.

Discussion

The inhibitor IgG fraction used in this study revealed reactivity to sites within each FVIIIa subunit as judged by dot blots. Use of LC-MS/MS techniques coupled with IP resolved the multiple antibody-antigen interactions in this complex mixture, identifying an ensemble of

epitopes that mapped to all A and C domains of FVIII. Indeed, the LC-MS approach proved far superior to the affinity-directed mass spectrometry approach employing MALDI-TOF that we used in an earlier study to map the epitope of anti-A2 domain monoclonal antibody [12]. Only two of the 19 peptides identified by LC-MS were identified by MALDI, and this result emphasized the increased sensitivity and resolving power of the current method. Furthermore, unlike recent techniques using peptide arrays to map inhibitor epitopes [8], the enhanced sensitivity of the LC-MS methods eliminated the requirement to affinity purify the IgG using immobilized FVIII.

Consistent with numerous reports (see Ref. [1] for review), the majority of peptides we identified mapped to sequences within the FVIII A2 and C2 domains. The 10 peptides that mapped to the A2 domain (residues 373-714) represented 7 distinct, non-contiguous sequences and accounted for a significant fraction (29%, 100/341 residues) of this domain (Figure 4). C2 domain (residues 2170-2332) showed a similar density of epitopes with three peptides representing 20% (33/163 residues) of this domain. We observed significantly less representation of A1, A3 and C1 domains in the epitope map. In most cases, epitopes were defined by relatively short peptide sequences of 12 residues or less, and in all cases sequences were no longer than 15 residues. Thus this approach provided a high level of definition for the residues comprising these antibody-binding sites.

A number of the peptide sequences identified have been shown by various biochemical or immunological methods to be important for specific FVIII functions, thus antibody binding these sites would be predicted to impair and/or eliminate that function. Importantly, two peptides comprising A2 residues 474-487 and 499-509 were identified that overlap the epitope for the inhibitory monoclonal antibody 413 (residues 484-509; [9]). Inhibitors to this region block FXase activity in a non-competitive manner [16], likely by preventing interaction between the A2 subunit of FVIIIa and the protease domain of FIXa [17]. Furthermore, two apparent continuous epitopes represented by overlapping sequences 532-541 and 538-551, and 577-584 and 583-593 lie in close proximity to the 558-loop in the A2 subunit which comprises a FIXa-interactive site in FVIIIa [18].

The three C2 peptide sequences identified fall within or overlap two larger segments, residues 2181-2243 [10] and 2248-2312 [19], to which sites for inhibitors have been mapped. The high-resolution structure of the C2 domain predicts both hydrophobic and electrostatic interactions in binding FVIII to membrane [20]. We note that peptides 2216-2227 and 2240-2249 contain three of the four basic residues, Arg2220, Lys2227, and Lys2249 that contribute electrostatic interactions with phosphatidylserine. Furthermore, the former sequence includes Val2223, while the latter sequence lies in close proximity to residues Leu2250/Leu2251, which form two of the hydrophobic-interactive sites.

A final noteworthy sequence, residues 360-372, is contained within a1 segment separating A1 and A2 domains and overlaps with the inhibitory monoclonal antibody, C5 (residues 351-365 [21]). It has been suggested anti-a1 inhibitors block the conformational change that yields active FVIIIa following activation [22]. Furthermore, residues 337-372 form a FX-binding site [23]. Thus antibody binding at this site could interfere with procofactor activation and/or the subsequent capacity for the cofactor to interact with substrate for FXase.

The neutralizing potential of antibodies binding to other sequences identified in the immuno-precipitate is not clear based upon failure to co-localize with known sites of function. However, we note that each sequence contains multiple residues that when mutated, yield a hemophilic phenotype as identified in the Hemophilia A database (<http://hadb.org.uk>, [24]). Indeed, one peptide comprising residues 200-208 shows point

mutations at 6 of the 9 residues result in a hemophilia, suggesting a potential functional role for this site. Given the lack of functional information regarding these sequences, we speculate that these epitopes minimally represent sites for non-neutralizing antibodies, which could potentially facilitate clearance of the FVIII protein.

In conclusion, we employed immuno-precipitations coupled with the high resolving power and sensitivity of LC-MS techniques to obtain an epitope map for the IgG fraction prepared from a high-titer FVIII inhibitor plasma. While some limitations with this method exist, such as gaps in the sequence coverage and potential proteolytic destruction of the epitope, these concerns were minimized with use of more than one protease. Advantages of the current approach include a level of sensitivity that eliminates the need to enrich the antibody pool for anti-FVIII antibodies and results in fine-point mapping the epitope to a relatively short sequence length. Thus the present technique complements other established epitope mapping protocols, which in general represent more labor-intensive methodologies and suffer from their own particular limitations. Within this inhibitor plasma we identified a population of epitopes for neutralizing and likely non-neutralizing antibodies that map to all A and C domains.

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[illegible]

Figure 1. Coverage maps of FVIII heavy chain (A; residues 1-740) and light chain (B; residues 1649-2332)

Coverage maps indicate peptides identified by LC-MS following digestion with trypsin (solid line) or chymotrypsin (dashed line). The map developed in panel A represents data derived from separate digests of the FVIIIa A1 (residues 1-372) and A2 (residues 373-740) subunits.

Figure 2. Dot blots of FVIIIa subunits reacted with the inhibitor IgG

Indicated amounts of purified FVIIIa A1, A2 and A3C1C2 subunits were spotted on PVDF and reacted with the inhibitor IgG as described in Methods. No reaction was observed in the absence of FVIII subunit or when FVIII was replaced with BSA.

Figure 3. LC-MS/MS analysis of FVIII light chain peptides following digestion with chymotrypsin and IP

(A) C18 reverse phase HPLC separation of a chymotryptic digest of light chain with base peak detection, producing a relative intensity signal over the 80 minute liquid chromatography run. The *arrow* indicates the 18.4 minute elution peak shown to contain the peptide LISSSQDGHQW, with the mass-to-charge ratio equal to 629.6 m/z (indicated by an *asterisk*). (B) Full MS spectrum at 18.4 minutes from the total protein digest from panel A. (C) Full MS spectrum at 18.8 minutes from an LC-MS/MS run with immunoprecipitated FVIII peptides. The *arrow* indicates the peptide isolated and fragmented for MS/MS analysis. The Y-axis in panels A-C represents relative abundance. (D) MS/MS fragmentation spectrum of the peptide LISSSQDGHQW from the antibody bound sample, described in panel C, *arrow*. Inset at the top shows the mapped peptide sequence and indicates the mapped b-ions and experimentally mapped y-ions.

Figure 4. FVIII structure indicating epitope sequences

The FVIII structure [25] is indicated in ribbon format depicting A1 (cyan), A2 (blue), A3 (yellow), C1 (dark green) and C2 (green) domains. Epitope sequences are shown in red, space-filling format.

Table 1

FVIII peptides identified by IP and LC-MS/MS

#	Position	Sequence ^J	Obs	Charge	Mr(expt)	Mr(calc)	δ (da)	Ion Score
1	52-59 ^C	VEFTDHL F	504.3090	2	1006.6034	1006.476	0.1	23
2	200-208 ^C	AVFDEGKSW	519.9650	2	1037.9154	1037.4818	0.4	31
3	360-372 ^T	FDDDNSPSFIQIR	777.6605	2	1553.3064	1552.7158	0.6	51
4	428-437 ^T	FMA YTDTEFK	626.9645	2	1251.9144	1251.5482	0.4	40
5	474-487 ^C	NIYPHGITDVRPLY	646.2.810	2	1290.5474	1290.7197	-0.2	47
6	499-509 ^C	KDFPILPGEIF	638.3795	2	1274.7444	1274.6911	0.1	42
7	513-523 ^T	WTVTVEDGPTK	616.8995	2	1231.7844	1231.6085	0.2	46
8A	532-541 ^T	YYSSFVNMER	656.3155	2	1310.6164	1310.5601	0.1	52
8B	538-551 ^C	NMERDLASGLIGPL	793.1945	2	1584.3744	1583.8341	0.5	33
9A	577-584 ^C	SVFDENRSW	570.1340	2	1138.2534	1138.5034	-0.3	22
9B	583-593 ^{T,2}	SWYLTENIQR	655.4300	2	1308.8454	1308.6462	0.2	47
10A	621-627 ^C	VFDSLQL	821.3280	1	820.3207	820.4331	-0.1	20
10B	628-637 ^C	SVCLHEVAYW	632.6110	2	1263.2074	1262.5754	0.6	27
11	1757-1762 ^C	GLLGPY	619.3920	1	618.3847	618.3377	0.05	23
12	1881-1889 ^C	TIFDETKSW	564.1525	2	1126.2904	1125.5342	0.8	17
13	2137-2147 ^{T,2}	HNIFNPPIAR	646.2810	2	1290.5474	1290.7197	-0.2	47
14	2216-2227 ^T	SNAWRPQVNNPK	470.7920	3	1409.3542	1409.7164	-0.4	16
15	2240-2249 ^T	VTGVTTQGVK	495.6295	2	989.2444	989.5553	0.7	56
16	2261-2271 ^C	LISSQDGHQW	629.3615	2	1256.7084	1256.5786	0.1	70

^T Peptide derived from tryptic digestion

^C Peptide derived from chymotryptic digestion

^J Single letter designation for amino acid residues is used

² Peptide also detected by affinity-directed MALDI-TOF mass spectrometry