Pleckstrin Homology Domains in Cell Signaling

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Pleckstrin homology (PH) domains are a structurally conserved family that is associated with many regulatory pathways within the cell. In particular, PH domains are found in many proteins involved in signal transduction such as phospholipases, GTPase-regulating proteins and protein kinases, but also in cytoskeletal proteins such as spectrin and syntrophin. They generally function as regulated membrane-binding modules that bind to inositol lipids and respond to upstream signals by targeting the host proteins to the correct cellular sites. In some cases, PH domains can directly control enzymatic activity of adjacent kinase or nucleotide exchange domains [1].

Crystal structures of the PH domains from PLC-d1 and spectrin have been determined in complex with lns(1,4,5)P3 and that of the Btk PH domain in complex with Ins(1,3,4,5)P4 [see 1, 2]. The phospholipid-binding site is not structurally conserved in all cases. The spectrin domain binds phospholipid between b1/b2 and b5/b6 loops, whereas the ligand binds on the opposite side of the b1/b2 loop, between this and the b3/b4 loop in the other PH domains.

Many studies have shown that some PH domains are specific for 3-phosphorylated inositol derivatives and therefore represent possible downstream targets of phosphoinositide 3-kinase (PI 3-kinase). PI 3-kinase is activated by receptor tyrosine kinases and G-protein coupled receptors in response to a wide range of cellular stimuli and produces Ptdlns(3,4)P2 and Ptdlns (3,4,5)P3 on the inner leaflet of the plasma membrane. Several PH domains, such as those of Akt, Btk, PLCg, and ARNO, have been shown to translocate to the plasma membrane following PI 3-kinase activation.

Details of molecular mechanism of inositol binding can be gained from mutations in the Btk PH domain. These mutations lead to a defect in the maturation of B cells, resulting in a severe human immunodeficiency known as X-linked agammaglobulinemia (XLA). The mutations may be grouped depending on their effect on binding. Many mutations directly perturb the inositol phosphate-binding site, whereas others have a more indirect effect. The PH domain mutants in which Arg28 is substituted with either a cysteine or a histidine remove a positive charge within the binding pocket and thus have a strongly reduced affinity for lns(1,3,4,5)P4.

Mutations located on the domain surface outside the binding pocket highlight the role of electrostatics in binding. Mutation of Lys 19, which is not in direct contact with the ligand, to a glutamate reverses a charge and significantly decreases the positive potential around the binding site. The resulting decreased affinity for the negatively charged inner surface of the cell membrane appears to be sufficient for the disease phenotype. Analogously, a gain of function mutant, E41K, enhances the positive potential. This hinders the removal of Btk from the membrane surface and hence its deactivation, leading to a transformation of cultured cells [2].

PH domains can regulate the activity of proteins not only by targeting to the correct subcellular location but recent data also suggest a direct allosteric activation. An example is the regulation of nucleotide exchange activity in the Dbl protein family by PH domains. Their structural hallmark is that the Dbl homology (DH) domain is immediately followed by a PH domain. The DH domain is a specific guanine nucleotide exchange factor (GEF) for the Rho family of small GTPases, which are involved in regulation of the actin cytoskeleton. Several studies show that the binding of inositol phosphates to the PH domain can modulate the nucleotide exchange activity of the adjacent DH domain. In addition, in many proteins the Sec7 domains that are GEFs acting on the Arf-class of small GTPases ad, have an adjacent PH domain. GRP1, ARNO, and cytohesin-1 that belong to this family specifically bind to 3-phosphorylated inositols and localize to the membrane following activation of PI 3-kinase [reviewed in 1].

A peculiar feature of the PH domain structures is the strong polarization of charges [3]. In the lipid-binding domains, the face of the molecule interacting with the inositol phosphate is surrounded by a strong positive potential. Electrostatic effects are probably involved in orientation of the molecule towards the membrane and can be a major determinant in binding. Electrostatic properties are in general well conserved within the PH domain family. However, in comparison to the domains that bind phospholipids, a small number of PH domains shows reversed polarization or an overall negative potential [1, 3]. That is inconsistent with binding to negatively charged phospholipids.

It is unclear whether all the DH-PH domains are regulated by phosphoinositides. Roughly half of the domains in the DH-PH family have the electrostatic properties typical for the PH domains with positively charged (lipid-) binding surface. For instance, the PH domain from Sos shows a similar potential profile to the main group of PH domains, and it is reported to bind phospholipids. In contrast, five out of seven PH domains within the whole family that are predicted to have a reversed potential with negative charge around the canonical lipid-binding site, are linked to a DH domain [3]. It is not likely that these domains bind acidic phospholipids.

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Identification of MHC-associated Peptides from Trypanosoma cruzi *Infected Cells by Liquid Chromatography/Tandem Mass Spectrometry*

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Trypanosoma cruzi is an intracellular protozoan parasite and the causative agent of Chagas' disease. This devastating disease affects an estimated 15 to 20 million persons in Latin America, and the chronic form of the infection can manifest itself as a fatal cardiomyopathy [1]. *T. cruzi* amastigotes reside in the cytoplasm of host cells where they release proteins that can enter the major histocompatibility complex (MHC) processing and presentation pathway [2]. MHC class I-bound peptides and the $CD8^+$ cytotoxic T lymphocytes (CTLs) that recognize the MHC-peptide complex are critical players in cell-mediated immunity to *T. cruzi* [3]. For this reason, the identification of parasite peptides that are presented by the MHC on the surface of infected host cells is an important prelude to the development of vaccines.

We have previously used a variety of techniques to identify targets of the CTL response in *T. cruzi*, including the screening of synthetic peptides encoded by previously cloned genes for MHC binding and CTL targeting activity, and the cloning of additional genes that we suspected would be CTL targets [4, 5]. While these considerable efforts were rewarded with the identification of three CTL target molecules, the discovery process involved long and expensive investigations. We sought to circumvent the necessity of this biologically intensive empiracle approach by the use of alternative analytical methods.

Hunt and coworkers have pioneered a method of identification of CTL target peptides [6, 7]. In this method, the fractionation of MHC-associated peptides using high-pressure liquid chromatography (HPLC), the identification of fractions containing relevant target peptides using standard CTL assays, and the sequencing of the target peptides using MS/MS are combined. The approach is facilitated by the iterative use of preparative reversed-phase HPLC in conjunction with the CTL assay to lower the complexity of the peptide mixture introduced to the mass spectrometer for sequencing [6, 7]. This method works well in instances where the number of potential disease-specific peptides is low and thus only a relatively few CTL lines must be generated and maintained for the CTL assays. However, because *T. cruzi* exhibits a higher level of polymorphism and a lower concentration of pathogen-related MHC peptides than myeloma cell lines, the effectiveness of CTL assay is limited. In fact, we were unable to stimulate CTLs with naturally derived peptides such that a significant signal-to-noise level could be achieved. However, we were able to detect and identify peptides that effectively stimulate CTLs at concentrations achieved using synthetic materials.

We have developed an alternative strategy for identifying targets of the CTL response that use information from the pathogen's genome to avoid the necessity of generating CTL lines for peptide identification [8]. In this approach, genome sequences from infecting agents are scanned for stretches of amino acids that match a particular MHC binding motif. The identified peptide sequences are then tabulated, and their masses are calculated and compared to those of peptides isolated from pathogen-infected host cells. Peptides in the pool with masses matching those in the database can be sequenced by MS/MS to determine their identity. Using this approach we were able to confirm the processing and presentation of two *T. cruzi* proteins by the MHC class I pathway. The approach does not necessitate that the parent protein of the peptide be known in advance, or that genome sequence information be complete in nature. In the example presented here, the genome sequence was far from complete. Experimentally, the method does demand that genetically characterized models with a known MHC binding motif be used and that the pathogen of interest is also known. These data suggest that a rigorous automated sequencing approach employing two-dimensional separations in conjunction with MS/MS and bioinformatics is a feasible approach to identify pathogen gene products of immunological interest when CTL assay is rendered experimentally impossible. Such an approach may provide a systematic method to identify parent proteins (vaccine candidates), independent of biological assays, for a variety of infectious disease systems.

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Mass Spectrometry in Protein Studies from Genome to Function

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The triumphal progress of DNA sequencing

Advanced technology for determination of DNA sequences has become widely available in the last decade and has been used for sequencing cDNA and entire genomes from a variety of organisms ranging from viruses to human. As a consequence, the amount of DNA sequence information entered in publicly accessible databases has increased exponentially in the last decade. This growth has by far exceeded the growth of sequences entered in databases based on protein sequencing. In addition to the genomic sequencing, large-scale partial cDNA sequencing has resulted in another set of data, the so-called expressed sequence tags containing stretches of sequence from a large number of genes from a variety of organisms. The genomic sequences, however, only provide information about the potencial of the selected microorganisms and cell types but do not reflect the actual situation at any given moment, i.e. which proteins are expressed and how they are modified. cDNA sequences or the incomplete ESTs gives information on the actually expressed proteins, but no information on processing and secondary information. Therefore, studies of the proteins will never be obsolete.

Mass spectrometry has conquered protein analysis

Independently, but coinciding in time, mass spectrometric analysis has undergone an equally dramatic development. From being an analytical tool for analysis of small volatile molecules, new ionization methods, especially electrospray ionization (ESI) [1] and matrix assisted laser desorption/ionization (MALDI) [2], have increased the accessible mass range to include nearly all proteins. Mass accuracy and sensitivity have been improved to routinely allow molecular mass determination on the 100 ppm level or better of peptides and proteins available in only mid to low femtomole amounts [3]. Mass spectrometry (MS) has been proven ideal for analysis of peptide and protein mixtures and partial or complete sequence information can be generated from the single components in such mixtures by the so-called MS/MS techniques. In addition, MS is the ideal technique for analysis of post translational modifications in proteins [4], thus being the perfect complement to DNA sequencing.

Proteome analysis: the next step after the genome

Once a genome is sequenced, then the next natural step is analysis of the proteome, which as defined by Wilkins *et al.* [5] represent: "The total protein complement expressed by an organism, a cell or a tissue type". Proteome analysis involves two essential steps. Firstly, separation and visualization of the proteins and, secondly, identification of the proteins relative to the genomic sequence, if known. 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is presently the only technique for separation of all or the majority of the proteins from a given cell type. Identification of the proteins is now routinely carried out by mass spectrometry after proteolytic digestion of the proteins in the gel. This can be done either based on peptide maps produced by MALDI MS or partial sequences produced by ESI MS (For a complete strategy see: Shevchenko *et al.* [6], Jensen *et al.* [3]. Of these techniques peptide mapping by MALDI MS is the simplest and most sensitive, whereas the sequence based techniques are more specific. Partial sequences also often allow identification in cases where only partial protein sequence information is available, e.g., in EST databases [7]. Upon positive identification, the corresponding cDNA can then be ordered and sequenced.

Characterization of secondary modifications is essential

Once a protein is identified, then the next obvious questions are: Are the identified proteins post translationally modified and if so, how? If the purified protein is available, then the strategy is to compare its molecular mass determined by MS with that calculated based on the DNA sequence. If these masses are different, then the modified sites and the types of modification are identified based on mass spectrometric peptide mapping if relevant supplemented with MS/MS of relevant peptide ions or degradation with appropriate enzymes, e.g., glycosidases or phosphatases [8, 9]. If the proteins are only available as spots or bands in electrophoretic gels, then it is often not possible to determine the molecular mass of the intact protein, and characterization of post translational modifications must rely on peptide mapping before and after enzymatic treatments and when appropriate MS/MS. In such cases it is essential to obtain complete or very high sequence coverage in the peptide maps [10, 11].

Studies of protein interaction and higher-order structures

The acceptance of mass spectrometry as a tool for studies of protein interaction and protein higher-order structures is gradually increasing [4]. Interaction screening based on "affinity fishing" followed by mass spectrometric identification of the bound proteins shows great promise [12]. Studies of protein conformation by deuterium exchange, of surface exposed residues by specific labeling or limited proteolysis, and of the interaction interface by cross-linking, although not competitive with techniques like X ray crystallography and NMR spectrometry in terms of amount of detailed information, can yield valuable information on much smaller protein quantities.

Conclusion

Mass spectrometry is now a viable tool on all levels in studies of proteins from genome to function.

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Modelling of Chimeric b-galactosidase Antigenic Fusion Proteins

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Introduction

A new principle for antibody detection has been proven to have a high potential for the development of faster analytical tests. lt has been shown that the insertion of antigenic peptides of the foot-and-mouth disease virus (FMDV) into specific sites of the bacterial enzyme β-galactosidase causes a severe reduction of the activity of this enzyme which can be recovered upon antibody binding to the inserted antigenic peptide [1]. In principle, the recovery of β-galactosidase activity upon antigen-antibody binding allows the detection of antibodies by simple colorimetric quantification of β-galactosidase activity in a homogeneous test system, involving only the antigenic fusion protein, a chromogenic substrate and a serum sample for testing.

The lack of three-dimensional structural information on the chimeric proteins renders their modelling requisite to understand the chimeric enzyme inactivation and reactivation upon antibody binding at the molecular level. For this purpose, we have determined models of chimeric β-galactosidase by comparative modelling and structure prediction techniques.

Materials and Methods

The 2D structures are predicted using the algorithm Fugue [2], while the 3D structures are calculated using the comparative modelling program Modeller5 [3].

Results and Discussion

Construction of the antigenic peptide at position 278 of the β-galactosidase has been performed since the construct is known to display high antigenicity and reactivation upon antibody binding to whole antibodies, but not to a single Fab fragment. It involves three main steps that will constitute a protocol to be used for other constructs.

1. To model the most probable conformations of the antigenic peptide to be inserted exploiting structural data either from X ray diffraction experiments or from secondary structure predictions. Several X ray structures of the whole viral proteins are available. The only structures, however, that show a well-defined conformation of the antigenic peptide are those of a mutant virus lacking one disulfide bridge (serotype O) and those of a short peptide (13 aa, serotype C) in interaction with SD6 antibody. Both experimental and prediction data agree on the local conformation: the segment before the highly conserved triplet Arg-Gly-Asp, which is involved in the recognition process, is in an extended conformation and the segment beyond adopts a helical conformation. However, the two experimental X ray structures show a relative orientation of the two segments that markedly differs. To model the peptide (27 aa) from serotype C with comparative modelling, only the serotype O structure is used as it displays a peptide sequence long enough to model the insertion even though it presents the drawback of a non-entirely homologous sequence (serotype O versus C).

2. To properly model the 3D structures of the peptide-inserted tetrameric β-galactosidase using β-galactosidase X ray structure, secondary structure predictions on the chimeric sequence and comparative modelling. Two- and four-residue lengths are chosen to anchor the peptide onto the enzyme so as to produce different potential orientations of the peptide at the enzyme surface. Overall 110 3D dimeric models are generated, 55 for each anchoring mode. A clustering procedure based on the computed root mean square deviation of the backbone atom coordinates is applied on the models. For example, with the 2-residue anchoring mode, 23 clusters are obtained for a rms cutoff of 1.5Å. As residues in the vicinity of the insertion site in the wild-type protein are known to interact with the active site of the facing monomer, conformational analysis of the selected models is performed to detect interactions between the inserted peptide in one monomer with active site residues of the other that could account for partial inactivation of the chimeric protein as experimentally observed. Molecular dynamics simulations are performed on the dimeric models to improve the conformational sampling of the inserted fragment accounting for its flexibility.

3. To model the interaction of the chimeric enzyme with the antibodies. The modelled dimers that display a solvent accessibility surface area large enough to bind the antibody are selected. Tetramers are built to test their ability to bind an antibody molecule.

The studies are currently extended to model other chimeric proteins carrying antigenic FMDV inserted at different positions. All models should help, in the one hand, to interpret and rationalize the large wealth of experimental data previously obtained. In the second hand, they should permit to guide new experiments on inserted-peptide enzymes and more particularly on new recombinant β-galactosidase HIV antigenic fusion proteins as enzymatic probes for anti-HIV antibody detection in HIV infected individuals.

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Bispecific Antibody-based Ultrasensitive Immunoassays

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Bispecific MAbs have non-identical paratopes unlike traditional monospecific MAbs and can be generated chemically, by somatic fusion of two hybridomas or by genetic engineering methods [1]. Bispecific antibodies allow the generation of the highest specific activity immunoprobes, wherein every antibody molecule can be labeled uniformly with the enzyme, as a label. In the present study we have developed bsMAbs against prostate specific antigen (PSA) and M13 phage as a model virus (Figure 1). The second arm in each case was specific to alkaline phosphatase (AP).

The major advances in immunoassays have focused on specificity, sensitivity, speed and convenience. New diagnostic markers have allowed earlier therapeutic intervention than was previously possible. The ability to quantify the analyte concentration in the ultrasensitive range has potential to identify residual and recurring disease several months and even years prior to their estimation by conventional immunoassay procedures. Among the ultrasensitive methods described, the technique of enzyme amplifications is the most sensitive method [2] which is capable of detecting as little as 0.01 attamole (amol) of AP [3]. The technique of enzyme amplification depends on an enzyme label giving rise to a catalytic intermediate that further amplifies the detectable signal. NADP+ for example as the primary substrate can be cleaved to NAD+ by AP. The dephosphorylated cofactor then enters a highly specific redox cycle, where it is reduced by NAD+ specific alcohol dehydrogenase. The oxidized form is regenerated by diaphorase with the concomitant reduction of *p*-iodonitrotetrazolium violet (INT-violet) reagent to an intensely purple formazan dye. The oxidized form is continuously cycled with the formation of detectable product with every turn of the cycle [4].

PSA is a 30-kD glycoprotein with chymotrypsin-like protease activity produced primarily by the epithelial cells of the prostate gland [5, 6]. Clinically, estimation of PSA levels allows early detection of prostate cancer and provides a way to monitor the treatment response and predict disease recurrence [7]. A number of clinical studies have shown that patients with radical prostatectomy have PSA concentrations < 0.1 µg/L and in most cases < 0.02 µg/L. These levels are undetectable by convencional assays which have a lower limit of detection of 0.1–0.4 µg/L [8]. Availability of ultrasensitive PSA assays may enhance our ability, detect the emergence of micrometastasis which are below the threshold of current assays.

Detection of virarl antigens and detection of the viral load present in body fluids is a desirable diagnostic or monitoring tool for many infectious diseases. In case of HIV infection, an HIV antigen assay could be used for measuring viral load to determine the prognosis of the infected individual and to monitor the effectiveness of the antiviral therapy [9]. In an effort to develop highly sensitive ELISA as a routine viral screening assay or viral load assay, phage M13 was chosen as a model virus to demonstrate feasibility since they were relatively safe and are conveniently available.

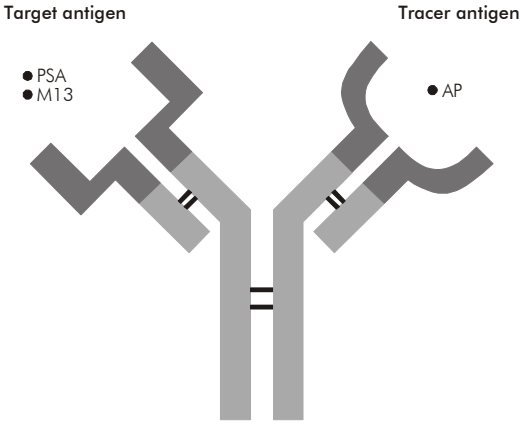

Figure 1. Bispecific antibodies.

This report describes the development of bispecific antibody based immunoassays for PSA and M13 phage coupled with enzyme amplified ELISA to achieve ultrasensitive detection of analytes.

We optimized various parameters of the bispecific assay to achieve the lowest detection limit. A sandwich assay format was used wherein a monospecific solid phase antibody was used to capture the antigen (PSA or M13). Subsequently, the corresponding bsMAb was added along with excess AP. Parallel assays were performed using either *p*-nitrophenyl-phosphate for conventional assay or an NADP based cyclic amplification reaction [4]. The results show that in the two examples investigated, we were able to demonstrate ultrasensitive detection of the two antigens which correspond to 250fin PSA/mL and 100 M13 particles or 2.3fg of coat protein (Figure 2).

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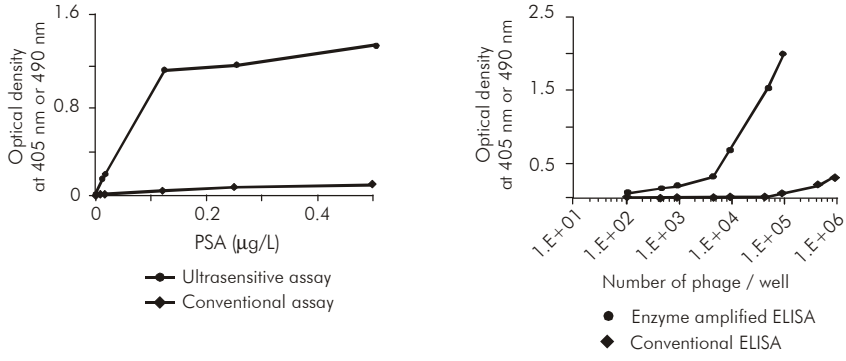

Figure 2. Comparison of conventional and enzyme amplification ELISA for detection of PSA and M13 phage.

Primary and Secondary Structure in Antibody Recognition of a Core Protein Epitope from Equine Infectious Anemia Virus

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*I*ntroduction

The major core protein of EIAV, p26, is one of the primary immunogenic structural proteins during persistent infection and is highly conserved among antigenically variant viral isolates. The detection of antibodies to p26 is the basis of the agar immunodiffusion test (AGID) [1].

The present work was started to determine minimal size epitopes within the C-terminal part of p26. We report our findings about the effect of conformation as an essential requirement for antibody recognition using elongated cycled peptides containing an identified epitope.

*M*aterials and Methods

Peptide synthesis by the Spot Method

Overlapping hexapeptides spanning the C-terminal region of p26, region 277-359 were synthesized on cellulose membranes chemically derivatized with spots of βAla-βAla anchor for the preparation of immobilized peptides. Assembly of the peptides was carried out using Fmoc-chemistry essentially as described [2].

Antibody-binding assay

lt was done with Alkaline Phosphatase (AP)-conjugated as secondary antibody, and the colour reaction was developed by a solution containing BCIP and MTT.

Manual peptide synthesis

A sequence from region 319-346 of EIAV core protein named as p26-1, was manually synthesized on solid phase using Fmoc chemical strategies, purified by semipreparative reverse-phase HPLC (Gilson System) and characterized by FAB-EM.

The thiol groups oxidation was carried out with iodine. The completion of the reaction was monitored by Ellman test.

Antibody binding to the linear and cyclic peptide

lt was identified by an indirect enzyme-linked immunosorbent, assay (ELISA).

Circular Dichroism (CD) measurements

CD spectra were recorded in a Jasco J-720 spectropolarimeter over the wavelength range of 195-250 nm. Measurements were made on peptide samples in the concentration range of 0.3-0.5 mg/mL in 1 mm path length quartz cuvettes. CD measurements were made at 20 ºC, in deionized water.

*R*esults and Discussion

Analysing the series of overlapping hexapeptides recognized by a pool of polyclonal sera of EIAV infected horses, several reactive sequences containing the common epitope MYACRD were identified.

An elongated peptide containing the native sequence from residues 319-346 named as p26-1 (ANEECR NANRHLRPEDTLEEKMYACRDIG) was prepared.

The CD spectrum of p26-1 linear and cyclic showed two minima next to 207 and 223 nm, but presented a different intensity between them. The following secondary structure fractions were calculated from SelCon Program: -p26-1 linear: 9% helix components, 41% β sheet components, 17% turn and 31% unordered; -p26-1 cyclic: 100% helix [3].

A significant conformational change was observed when the peptide was cycled by a bridge between Cys323 and Cys343 that correlated with an improved ability of the cyclic peptide to recognize antibodies in the ELISA test. These results suggest that the conformationally restricted peptide, adequately mimic the native structure of this portion of p26 core protein.

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Primary Structure Analysis of Variable Regions Encoding ABI and AB2 Antibodies from the N-*Glycolyl Ganglioside System*

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Introduction

We have previously reported the generation of five anti-idiotype monoclonal antibodies against the *N*-glycolyl containing ganglioside-specific monoclonal antibody, P3. These AB2 monoclonal antibodies have been classified as AB2g according to its inability to induce antigen-independent responses against *N*-glycolyl containing gangliosides even when they all block ABI-AG interaction in a dose-dependent manner [1]. In this report we described the nucleotide and amino acid sequences from the heavy and light chains variable regions of P3, and two anti-idiotype antibodies, 1E10 and 3B11. Our data suggest a correlation between primary structures and immunochemical properties.

Materials and Methods

Total RNA was prepared from hybridoma cells obtained in our laboratory [1, 2]. First-strand cDNA synthesis was performed with primers specific for murine heavy and kappa chains constant regions. Variable regions were amplified by the polymerase chain reaction (PCR) using previous primers and framework 1 (FRW1)-specific primers, cloned in M13mp19 vector (Pharmacia) and sequenced by the dideoxy method (T7 sequencing kit, Pharmacia). Individual VH and VL sequences were searched against the EMBL/Genbank database for sequence homology with known murine genes. The JK and JH regions sequenced were compared with the five known murine JK genes and with the four known murine JH genes [3]. D region nucleotides were identified after alignment of CDR3H nucleotide sequences with known murine D minigenes [3].

Results and Discussion

VH P3 is a member of the Q52 (VIH 11) gene family. The most related sequence to VH P3 is a cDNA sequence of asws 1 (IgG2a, k), a mouse antibody generated in A.SW (H-2s) strain with specificity to U3 and U8 ribonucleoproteic particles [4]. There are only two nucleotide differences between P3 and asws 1 (codons 2 to 94), leading to only one amino acid replacement. Analysis of differences at both positions and comparison with previous reported sequences strongly suggest that VH P3 represent a new putative germline gene from the VH Q52 family. In CDR3, P3 has 14 amino acids and JH3 is used. The CDR3H of P3 MAb have a seven nucleotide-long D region from the DSP2 family. N nucleotide additions can be identified at both VH-D and D-JH junctions. VH P3 has an unusually extensive 3N region with 15 nucleotides. The high level of N nucleotides in this CDR3H explains its elevated content of infrequent amino acids. This feature could explain the strong immunogenicity of this MAb in syngeneic model, an aspect that distinguishes this MAb from others studied in our group [1]. The VH 1E10 is a member of the J558 family and is almost identical to that present in H35-C7, a hibridoma generated in Balb/c making antibody against the influenza virus hemagglutinin [5]. This strongly suggests that 1E10 and H35-C7 use the same germline VH gene. VH 1E10 has a 12 amino acid-long CDR3H, and its D region seems to result from recombination of DSP2.2 and DSP2 minigenes. The JH2 segment is used. The most related sequence to VH 3B11 appears in cDNA sequence of MRB9, a polyreactive murine monoclonal antibody of IgM isotype that binds to purified total histones [6]. Both sequences share a 95% homology with differences in 11 nucleotide positions (codons 1 to 94). Analysis of the distribution of replacement versus silent putative mutations and comparison with previous reported sequences shows that VH 3B11 has extensive somatic mutations. 3B11 has a 9 amino acid-long CDR3 and JH3 used.

Even when different VH gene segments contribute to the variable regions of 1E10 and 3B11, Ab2 shares a homologous sequence in the heavy chain CDR3 enriched in acidic residues. This sequence homology is based on homologous N nucleotide additions at VH-D junction and the use of the same D gene, DSP2.2, in the same reading frame. On the other hand, a higher level of basic than acidic amino acid residues was found in the CDRs of Ab1 P3. These findings suggest an involvement of residues in the motif shared by both Ab2 in the recognition of P3 idiotype.

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