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Identification of cation-independent mannose 6-phosphate receptor/insulin-like growth factor type-2 receptor as a novel target of autoantibodies

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SUMMARY

Two human monoclonal autoantibodies, B-33 and B-24, were generated from the B cells of a patient with scleroderma. Both monoclonal antibodies (mAbs) were composed of μ and λ chains, and recognized cytoplasmic vesicular structures by indirect immunofluorescence on Hep-2 cell line slides, although mAb B-24 showed an additional diffuse cytoplasmic staining pattern. By Western blot, mAb B-24 exhibited a polyreactive-like binding pattern, whereas mAb B-33 failed to recognize any electroblotted Hep-2 antigen. The polyreactive versus monospecific behaviour of mAbs B-24 and B-33 was further confirmed by enzyme-linked immunosorbent assay (ELISA) with a variety of foreign and autoantigens. The N-terminal sequence of a protein band isolated by affinity chromatography with mAb B-33 was identical to that of cation-independent mannose 6-phosphate receptor (CI-MPR), also known as the insulin-like growth factor type-2 receptor (IGF-2R). Immunofluorescence experiments on Hep-2 cell line slides demonstrated a striking co-localization between the staining pattern exhibited by these mAbs and the pattern obtained using a goat anti-CI-MPR serum, indicating the recognition by B-24 and B-33 of a structure located predominantly in late endosomes. Sequence analysis of the V-region gene segments of B-33 and B-24 showed both to be identical, except for the existence of a point mutation in B-33 located in the H-complementarity-determining region 3 (H-CDR3) (position 100D), which produces a non-conservative replacement of Gly by Ser. This single replacement appears to be responsible for the dramatic change in reactivity of human mAb B-33. The data shown here provide new evidence of the critical role played by the H-CDR3 region in distinguishing a polyspecific from a monospecific antibody. A population study demonstrated the existence of immunoglobulin G (IgG) reactivity against CI-MPR/IGF-2R in serum specimens from five individuals with different pathological conditions, thus indicating that this molecule is a potential target for the human autoimmune response.

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Abbreviations: CDR, complementarity-determining region; CI-MPR, cation-independent mannose 6-phosphate receptor; CNBr, cyanogen bromide; DAB, diaminobenzidine; FR, framework; hLAMP-1, human lysosome-associated membrane glycoprotein-1; hLAMP-2, human lysosome-associated membrane glycoprotein-2; HRP, horseradish peroxidase; IGF-2, insulin-like growth factor-2; IGF-2R, insulin-like growth factor type-2 receptor; IIF, indirect immunofluorescence; PBST, 2% bovine serum albumin in phosphate-buffered saline containing 0·05% Tween-20 and 0·02% thimerosal; PDVF, polyvinylidene difluoride; TBS, tris-buffered saline; WGA, wheat germ agglutinin.

INTRODUCTION

A common feature of autoimmune diseases is a humoral immune response manifested by the presence of autoantibodies (autoAb) targeted against a wide spectrum of intracellular molecules that play the role of antigens (Ag). AutoAb have often been used as tools for studying the structure and function of their intracellular targets. For example, spontaneously occurring autoAb have been used to identify and clone chromatin, nucleolar, nuclear envelope and cytoplasmic proteins, and they have helped to elucidate the function of intracellular molecules and cellular processes, such as pre-mRNA splicing and DNA replication.[1] In addition, some autoAbs are highly specific and can be used as diagnostic markers for many autoimmune disease conditions. However, their main purpose still remains to be identified as they may be immunological imprints of events that induce the autoimmune response.

Although the mechanisms eliciting an autoimmune response are not yet fully understood, autoAbs rely on the

use of a relatively small set of immunoglobulin V-region genes, some of them recurrently used in germline configuration by natural Abs.[2] Natural (or polyreactive) Abs are primarily immunoglobulin M (IgM) and bind with low affinity to a variety of Ags, including self-Ags.[3–5] Regarding autoAbs found in patients with autoimmune diseases, numerous studies have indicated that many of them derive from the same pool of V-genes as those encoding polyreactive Abs, although they are usually IgG and bear somatic mutations located mainly in complementarity-determining regions (CDR),[6,7] and also differ in their generally monoreactive and high-affinity profile. From these data, it has been suggested that these autoAbs derive from natural Abs by an Ag-driven maturation.

The cation-independent mannose 6-phosphate receptor (CI-MPR) is a highly conserved multifunctional protein that plays a central role in sorting lysosomal enzymes.[8] This sorting process is accomplished by the phosphomannosyl recognition system. The discovery that the CI-MPR and the insulin-like growth factor type-2 receptor (IGF-2R) are the same protein raised the interesting possibility that this receptor functions in two distinct biological processes: protein trafficking and transmembrane signal transduction.[9,10] Cell transfection experiments have provided evidence that CI-MPR/IGF-2R mediates the transport of newly synthetized acid hydrolases to a prelysosomal compartment, where its low pH induces the dissociation of the ligand, which is then packaged into a lysosome. The receptor then either returns to the Golgi to repeat the process or moves to the plasma membrane where it functions to internalize exogenous lysosomal enzymes or, in some instances, to mediate a transmembrane signalling event upon the binding of insulin-like growth factor-2 (IGF-2) [reviewed in 9].

The development of human monoclonal antibody (mAb) production has enabled the isolation of clones of autoreactive B lymphocytes, and constitutes an invaluable tool for dissection of the autoimmune response and investigation of the nature of the recognized autoAg.[11–13] In this study, we report the identification of CI-MPR/IGF-2R, as a novel autoAg, by human mAbs derived from peripheral blood lymphocytes (PBL) isolated from a single scleroderma patient. Furthermore, autoAbs that react with this structure were also detected in the serum of five individuals with different pathological conditions. The amino acid sequences of mAbs B-24 and B-33 were deduced from their nucleotide sequences and found to be identical, except for a single, non-conservative replacement of Gly by Ser in the H-CDR3 region of B-33.

MATERIALS AND METHODS

Production and cloning of heterohybridomas

Human mAbs were generated according to Campbell,[14] with minor modifications.[15] Briefly, PBL from a patient with scleroderma (C. S. S.) were isolated and then treated with L-leucyl-L-leucine ethyl ester (Leu-Leu-Ome) (Boehringer Mannheim, Mannheim, Germany)[16] prior to Epstein–Barr virus (EBV) transformation. B-cell lines were fused with the human–mouse heteromyeloma SHM-D33.[17] Supernatants of culture wells showing cell growth after 3–4 weeks were screened for autoAb activity by indirect immunofluorescence (IIF) on Hep-2 cell line slides. Positive cultures were cloned twice by limiting dilution, as described previously.[18] Selected lines were expanded and maintained in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom), 2 mM L-glutamine (Sigma, St Louis, MO), and 5 μg/ml gentamycin sulphate (Biochrom). H- and L-chain determination of mAb, as well as their quantification, were established by enzyme-linked immunosorbent assay (ELISA) using a double-sandwich method.

Sera, cell lines and antibodies

Serum was collected from patient C. S. S. By IIF this serum exhibited a typical anticentromere staining pattern on Hep-2 cell slides. Serum samples from five individuals with different pathological conditions (A. T. C., Raynaud's phenomenon; R. H. J., severe leukopenia; C. A. C., purpura; A. G. L., arthralgia; and L. S. Z., chronic hepatopathy) were obtained from our laboratory serum bank. Hep-2 (American Type Culture Collection [ATCC], Rockville, MD; CCL 23) cells were grown as described above. Anti-β-COP mAb M3A4, used to label Golgi structures, was kindly provided by Dr T. E. Kreis.[19] To label recycling and early endosomes[20] we utilized a commercially available mAb against the human transferrin receptor (anti-CD71–fluorescein isothiocyanate [FITC]; Becton-Dickinson, Mountain View, CA). The anticlathrin heavy chain mAb (Boehringer Mannheim) was used to label clathrin-coated vesicles.[21] A goat anti-CI-MPR serum to label endosomal structures was kindly provided by Dr K. von Figura.[22] mAbs H4A3 and H4B4 (anti-human lysosome-associated membrane glycoprotein-1 [anti-hLAMP-1] and anti-hLAMP-2, respectively), utilized to label lysosomes, were obtained from the Developmental Studies Hybridoma Bank (John Hopkins University School of Medicine, Baltimore, MD and the University of Iowa, Iowa City, IA).[23]

IIF lectin staining

IIF was performed on commercially available Hep-2 cell line slides (Kallestad, Chaska, MN). Ab labelling was carried out essentially as described previously.[24] Undiluted supernatants from clones B-24 and B-33, and appropriately diluted human serum specimens, were revealed with FITC- or tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-human IgM and IgG (Dako, Carpinteria, CA), respectively. Abs against marker proteins were appropriately diluted and revealed with their corresponding FITC-conjugated anti-species immuoglobulin (Dako). For lectin staining, cells were incubated with 0·5 mg/ml TRITC-conjugated wheat germ agglutinin (TRITC-WGA; Sigma) for 30 min at room temperature under humidified conditions. For comparative immunolocalization studies, cells were first incubated with human mAb B-33, then with anti-hLAMP-1, anti-hLAMP-2, anti-CI-MPR, or TRITC-WGA. This was followed by incubation with a mixture of TRITC-conjugated rabbit antihuman IgM and FITC-conjugated rabbit anti-mouse immunoglobulin, or FITC-conjugated rabbit anti-goat immunoglobulin (Dako). Slides were mounted in 90% glycerol in phosphate-buffered saline (PBS) and examined with an epifluorescence microscope (Carl Zeiss, Thornwood, NY).

Western blot

Immunoblotting was carried out by using extracts derived from Hep-2 cells.[12] Electrophoresis of cell lysates was performed under reducing conditions, in 10% (w/v) polyacrylamide gels,

according to Laemmli.[25] The separated proteins were electroblotted onto polyvinylidene difluoride membranes (PVDF; Immobilon-P; Millipore, Bedford, MA).[26] Unbound sites on the PVDF strips were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0·05% Tween-20 and 0·02% thimerosal (PBST) for 1 hr at room temperature, and then overlaid with appropriately diluted goat anti-CI-MPR serum or rabbit anti-bovine serum proteins (Dako) for 1 hr at room temperature. Human mAbs were used as undiluted heterohybridoma culture supernatants and incubated overnight at 4°. Then, the strips were washed thoroughly with PBST to remove any unbound Ab, and incubated with a 1 : 2000 dilution in PBST of appropriate alkaline phosphatase-conjugated secondary Ab (Dako). After washing, the colour was developed with bromochloroindolyl phosphate/nitro blue tetrazolium (Bio-Rad, Richmond, CA) in 0·1 M Tris, pH 9·5, containing 0·1 M NaCl and 5 mM $MgCl_2$.

ELISA for reactivity with multiple Ags

mAbs B-24 and B-33 were examined for their binding to diverse Ags. Detection of Ab activity against purified human IgG, nucleoprotamine, histone, salmon-sperm nuclei, bovine insulin, cardiolipin, phosphorylcholine, fibronectin, myeloperoxidase, rabbit thymus extract, human spleen extract, actin, cytokeratin, desmin, vimentin, myosin, tropomyosin, troponin, single-stranded DNA, double-stranded DNA, ribonucleic protein, Sm, SS-A/Ro and SS-B/La,[1] was performed by ELISA, as described previously.[4]

Ag purification and microsequencing

An affinity chromatography column was prepared by binding 2 mg of human mAb B-33, concentrated by ultrafiltration, to 1 mg of rabbit anti-human IgM (Dako) coupled to cyanogen bromide (CNBr)-activated Sepharose 4b beads (Pharmacia, Uppsala, Sweden). Hep-2 cells were biotin labelled (see below) and lysed. After Hep-2 cell lysate loading, the column was washed serially with 20 column volumes of extraction buffer (50 mM $Na_2B_4O_7$, 150 mM NaCl) and 10 volumes of 10 mM NaH_2PO_4, pH 6·8. Bound material was then eluted with 0·5 column volumes of 100 mM glycine, pH 2·5, and 1-ml fractions were collected in tubes containing 0·1 ml of neutralizing buffer (1 M NaH_2PO_4, pH 8·0). Aliquots of each fraction were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto PVDF membranes. The blots were blocked with 2% BSA in PBS and developed by incubation with appropriately diluted avidin–horseradish peroxidase (HRP; Bio-Rad). Bound avidin–HRP was visualized by incubating the blot with diaminobenzidine (DAB; 0·6 mg/ml of DAB in 0·05 M Tris buffer, pH 7·6, 0·03% H_2O_2).

A band with a $M_r > 200$ kDa, purified from unlabelled Hep-2 cell extracts with mAb B-33, was isolated from blots and processed for N-terminal microsequencing by CB Laboratories (Richmond, VA). Computer analysis of protein sequences was performed using the University of Wisconsin Genetics Group Sequence Analysis Software Package.[27]

Chromatography on mannose 6-phosphate affinity columns: dot-blot

The purification of MPR from Hep-2 cells was carried out using a commercially available matrix of mannose 6-phosphate coupled to agarose beads (Sigma). Briefly, unlabelled cell extracts were mixed with the agarose beads and incubated overnight at 4° with rotation. Beads were washed twice with lysis buffer (150 nM NaCl, 1% Nonidet P-40 [NP-40], 0·5% sodium deoxycholate, 0·1% SDS, 0·1 mg/ml phenylmethylsulphonyl fluoride [PMSF], 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM Tris, pH 7·5) and twice with Tris-buffered saline (TBS), by centrifugation at 1500 **g** for 10 min. The receptor was eluted under acidic conditions (100 mM glycine, pH 2·5). A small aliquot of the eluted sample was then processed for purity analysis by SDS–PAGE.

Aliquots of unlabelled samples purified either by affinity chromatography, using mAb B-33, or the mannose 6-phosphate-coupled agarose beads, were spotted onto nitrocellulose (Bio-Rad) using a Bio-Dot microfiltration unit (Bio-Rad). The membrane was then blocked with 3% BSA in PBS for 2 hr at room temperature and incubated with appropriately diluted antisera or undiluted human mAb. After washing with TBS, appropriate alkaline phosphatase-conjugated secondary Abs were added, and the membrane was then incubated for 1 hr at room temperature, washed and developed as described above for Western blotting.

Immunoprecipitation

IgG was purified from serum samples to immunoprecipitate biotin-labelled Hep-2 cell components. This was achieved by using the Cellular Labeling and Immunoprecipitation Kit (Boehringer Mannheim), following the manufacturer's instructions. Immunoprecipitated proteins were separated by SDS–PAGE, blotted onto a PVDF membrane and detected with the BM Chemiluminescent Blotting Kit (Boehringer Mannheim). A commercially available preparation of IgG purified from pooled normal human serum (Sigma) was included as a control.

Sequencing of V_H and V_L genes

Total RNA was extracted from 10^6 exponentially growing hybridoma cells by the guanidinium thiocyanate method.[28] First-strand cDNA was synthesized from 5 μg of total RNA with oligo(dT) as primer and the avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim). Amplification of cDNA was performed with family-based back primers, specific to the 5′ region of each V_H family, as described by Marks *et al.*,[29] and a Cμ or Cλ oligonucleotide to prime from the 3′ end. For λ chains the Vλ 5′-specific primers were: VλI: 5′-CAGTCTGTGTTGACGCAGCCGCC-3′; VλII: 5′-CAGTCTGCCCTGATTCAGCCTCC-3′; and VλIII: 5′-CCTATGAGCTGACTCAGCCACC-3′. DNA fragments were subcloned into the *Eco*RV restriction site of the pBluescript KS + cloning vector, as recommended by the manufacturer (Stratagene, La Jolla, CA).

DNA sequencing was carried out using the dideoxynucleotide technique of Sanger *et al.*[30] and T3 20-mer and KS 17-mer biotinylated primers (Stratagene). After electrophoresis, DNA was transferred to a positively charged nylon membrane (Tropilon Plus, Tropix, Bedford, MA) by capillary action and immobilized on the membrane by UV irradiation. Membranes were developed using the Seq-Light chemiluminescent DNA sequencing system (Tropix), following the manufacturer's instructions. Each V_H or V_L gene sequence was generated from three independent clones derived from an independent polymerase chain reaction (PCR). Computer

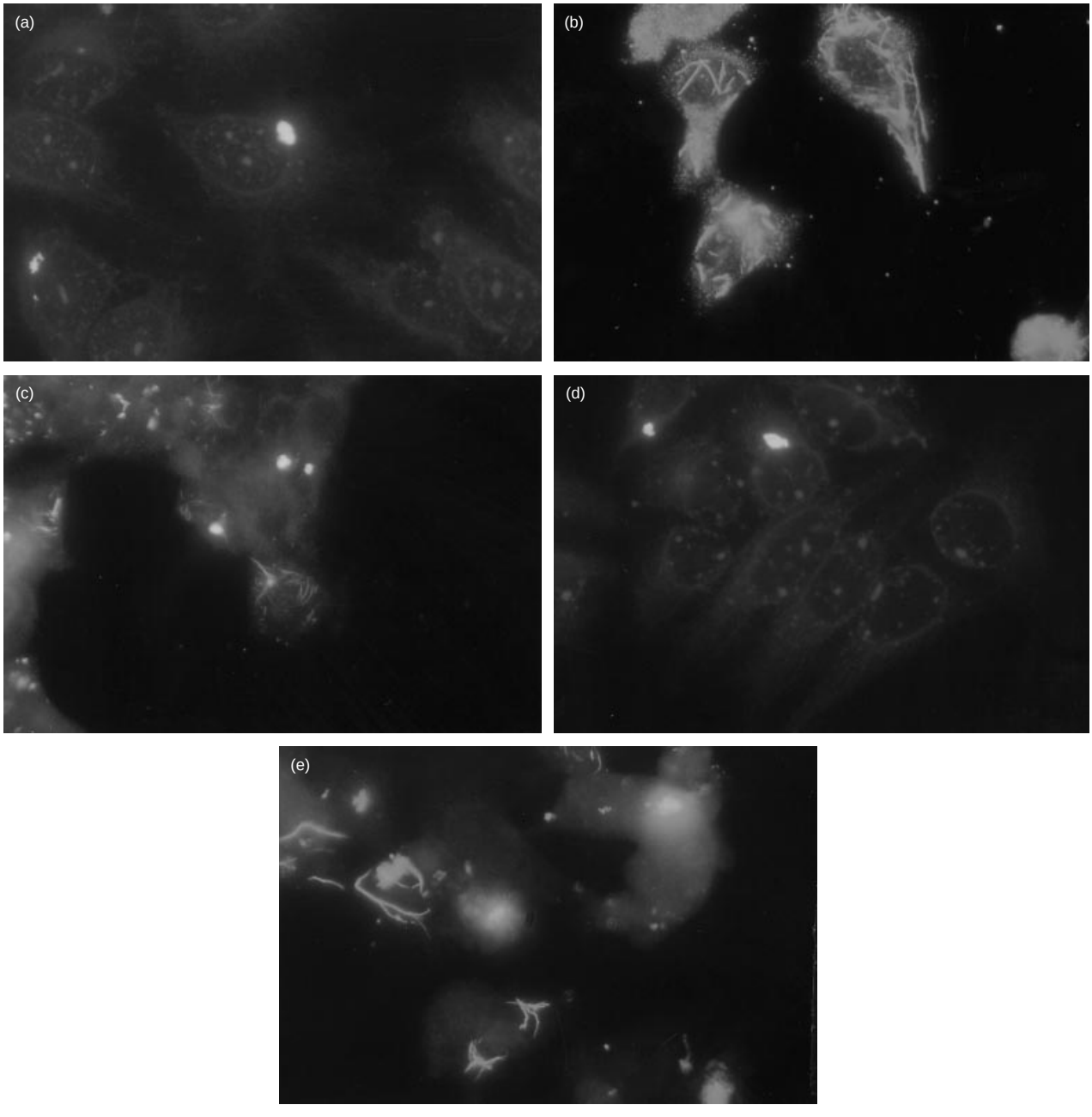

Figure 1. Immunofluorescence staining on Hep-2 cells. (a) (b) and (c) Monoclonal antibody (mAb) B-33. (d) and (e) Serum sample from patient A. T. C.

analysis of nucleic acid sequences was performed using the University of Wisconsin Genetics Group Sequence Analysis Software Package.[27]

RESULTS

Generation of human mAb

Positive clones of heterohybridomas were selected on the basis of reactivity by IIF on Hep-2 cell slides. As a result of cloning positive cultures, seven Ab-secreting clones were isolated (B-24-7, B-24-8, B-24-9, B-24-11, B-14, B-33q and B-33r), showing two different IIF staining patterns on Hep-2 cells (see below). One clone of each pattern was selected, expanded for Ab production and named B-33 and B-24, respectively. Isotype analysis showed that all clones secreted immunoglobulins composed of μ and λ chains.

Reactivity of human mAbs B-33 and B-24

mAbs B-33 and B-24 stained cytoplasmic vesicular structures on Hep-2 cells by IIF. These structures were different in size and their intracellular arrangement was also relatively heterogeneous (Fig. 1a). In some cells, an extensive tubular reticulum with swellings along its length was observed, often branching to form complex networks (Fig. 1b). This tubular reticulum was found throughout the cell cytoplasm, although not seeming to

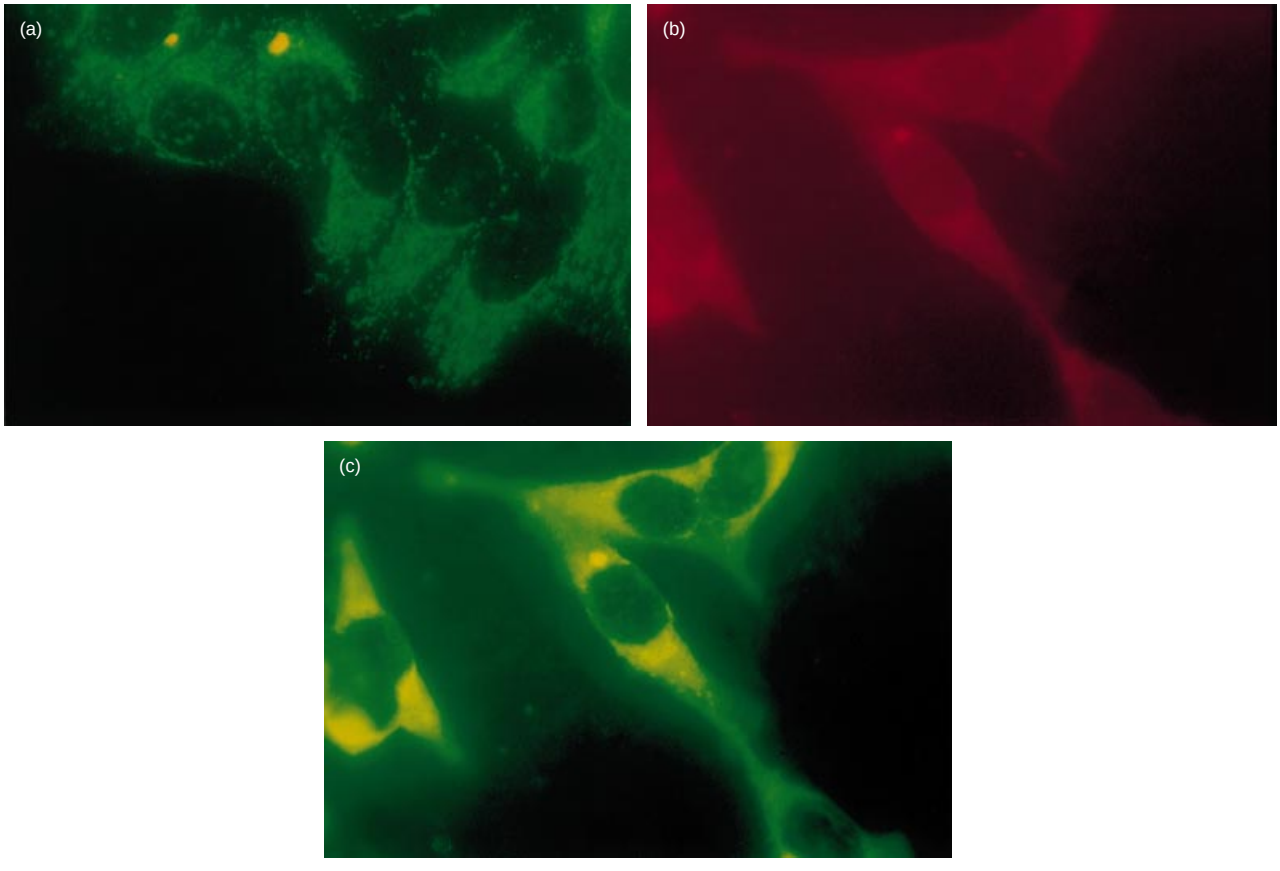

Figure 2. Double fluorescence staining on Hep-2 cells. (a) Monoclonal antibody (mAb) B-33 (orange staining), anti-human lysosome associated membrane glycoprotein-1 (anti-hLAMP-1) and hLAMP-2 antibody (green staining). (b) mAb B-33 (red staining). (c) Anti-cation-independent mannose 6-phosphate receptor (anti-CI-MPR) serum (green staining).

form a single, continuous organelle. The longer cisternae were frequently centriole-oriented, expanding sometimes from the peripheral cytoplasm, just below the plasma membrane, down into the pericentriolar area. Occasionally, diverse stages from vesicular to reticular structures were observed (Fig. 1c). Additionally, mAb B-24 showed a diffuse cytoplasmic staining pattern (data not shown). It is interesting to point out that this pattern was not observed with sera of the patient from which the mAbs were generated.

Five serum specimens (A. T. C., R. H. J., C. A. C., A. G. L. and L. S. Z.) exhibiting the same IIF pattern as B-33 on Hep-2 slides, were identified after daily routine screening for autoAb in our laboratory (Fig. 1d, 1e) in a time-period of $\approx$ 5 years ($\approx$ 25 000 sera were screened). These autoAbs belonged to IgG and IgM isotypes. A control population comprising 400 healthy individuals, as well as a well-characterized pathological population of patients with diverse autoimmune conditions, such as systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis, Sjögren syndrome, mixed connective tissue disease, etc., were found to be negative for this staining pattern.

The identity of the vesicular structure was established using different compartment markers: TRITC-WGA, used as a Golgi marker, gave a punctate perinuclear staining as well as the antiβ-COP mAb (data not shown). Neither anticlathrin nor antitransferrin receptor Abs recognized the vesicular structure stained with mAbs B-33 or B-24 (data not shown). Using anti-LAMP-1 and anti-LAMP-2 Abs, peripheral staining of the large vesicles recognized by mAbs B-33 and B-24 was observed (Fig. 2a). However, a striking co-localization of mAb B-33 and a goat antiserum against CI-MPR was clearly established (Fig. 2b, 2c). This finding indicates that mAb B-33 recognized a molecular structure located predominantly in the late endosomal compartment, with undetectable staining in other cellular compartments.

Testing the autoreactivity of mAb B-33 by immunoblotting on whole Hep-2 cell extracts revealed its inability to recognize any electroblotted Ag. On the contrary, mAb B-24 was able to recognize multiple components of the cell extract, exhibiting a banding pattern as described previously for polyreactive or natural Ab (data not shown).[3,4] To determine whether the pattern of reactivity of mAb B-24 was caused by polyreactivity or the recognition of an epitope shared by several different cell constituents, both mAbs were examined, by ELISA, for their binding to a wide panel of Ag. The results showed that mAb B-24 was able to react against actin, troponin, SS-B/La, rabbit thymus extract and human spleen extract, whereas B-33 failed to recognize any of the Ags tested.

Characterization of the autoAg

The Ag was purified by affinity chromatography of biotin-labelled Hep-2 cell extracts using mAb B-33. mAb B-24 was discarded in these experiments owing to its polyreactive

behaviour. Previously, by blocking the free amino groups of mAb B-33 with dinitrofluorobenzene, we determined that coupling to lysine residues by means of CNBr-activated Sepharose beads completely abrogated the immunoreactivity of this mAb. Therefore, we used rabbit anti-human IgM Ab coupled to a matrix of CNBr-activated Sepharose beads, avoiding covalent coupling as well as previous purification of mAb B-33. This substrate was used to bind mAb B-33 and further purify the Ag recognized by this mAb.

The eluted fraction was electrophoresed under reducing conditions and electroblotted onto PVDF membranes to develop the biotinylated proteins. Three main bands were identified with $M_r > 200$ kDa (band A), 45 kDa (band B) and < 45 kDa (band C), respectively (Fig. 3, strip a). Other background bands were visualized in the blot and considered to be contaminants. Band B was excluded for further experiments as it was recognized by Ab specific for bovine serum proteins (see strip b in Fig. 3). Bovine serum proteins are present in Hep-2 culture medium and could bind non-specifically to some chromatographic components. It is well known that the use of anti-immunoglobulin bridges between the column matrix and the specific Ab improves its immunoreactivity because covalent coupling could affect the residues involved in Ab binding. On the other hand, this strategy usually increases the background of the purified samples owing to cross-reactions, non-specific binding to the anti-immunoglobulin, as well as to the unavoidable cleavage of IgM by the acidic conditions of the elution.[31] To obtain greater insight into the identity of bands, an immunoblot of the purified sample was developed with the goat anti-CI-MPR serum. Interestingly, these Abs recognized not only band A but also band C, suggesting that band C is merely a breakdown product of band a (Fig. 3, strip x).

Once band A was considered to be the presumed autoAg, the N-terminus of this band was microsequenced. With as few as 30 pmol of the transblotted sample, six residues were unambiguously assigned and showed to be identical to those of CI-MPR. Furthermore, band A migrated to the expected position for CI-MPR on SDS–PAGE. The fact that CI-MPR bound multiple ligands containing a residue of mannose 6-phosphate,[32] in addition to the presence of the soluble portion of bovine CI-MPR and IGF-2 in FCS,[9] may explain the background obtained at the onset of its purification.

In order to confirm unequivocally the anti-CI-MPR reactivity, it was necessary to develop a procedure that allowed discrimination between contaminants and the target of our autoAb. To achieve this, we performed Western blotting with the sample purified using mAb B-33, as well as dot-blot assays with the sample purified using the phosphomannosyl resine, which captures both types of receptors (cation-independent and cation-dependent) by their binding site, thus avoiding the co-purification of mannose 6-phosphate-linked glycoproteins. As expected, results from immunoblotting were in agreement with previous experiments using whole cell extracts, that is the total absence of recognition of any component by B-33. This finding clearly indicates the lack of recognition of the denatured form of the Ag by mAb B-33. On the contrary, this mAb, as well as the five serum specimens exhibiting the same immunofluorescence staining pattern as B-33 on Hep-2 cells, recognized the receptor in the native state, as shown by dot-blot analysis of the sample purified with the phosphomannosyl resine, indicating that they react with a conformational epitope of the molecule disrupted in the SDS–PAGE. This was further supported by immunoprecipitation of a band of $M_r > 200$ kDa with the IgG purified from the serum samples (Fig. 4), not seen by Western blot on Hep-2 cell lysates (data not shown).

Sequence analysis of mAbs B-33 and B-24

Human mAbs B-33 and B-24 used a V_H3 gene segment that was 97% homologous to the germline gene DP-53,[33] which is the furthest functional segment from the J_H region. This segment differed from that of DP-53 in eight nucleotides – three of them were located in the CDR and produced the following

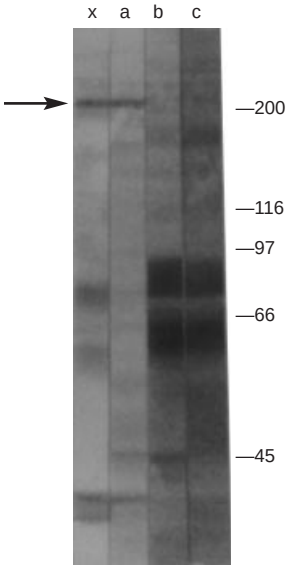

Figure 3. Western blot of the sample purified with monoclonal antibody (mAb) B-33 (band A is arrowed). Strip x, goat anti-cation-independent mannose 6-phosphate receptor (anti-CI-MPR) Ab; strip a, biotin-labelled proteins; strip b, anti-bovine serum proteins + anti-human immunoglobulin M (IgM); strip c, anti-human IgM.

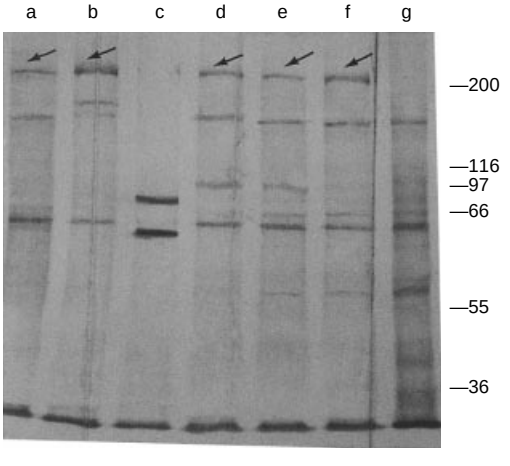

Figure 4. Immunoprecipitation of biotin-labeled Hep-2 proteins with immunoglobulin G (IgG) from autoimmune sera and pooled normal human IgG. Lanes: a, patient A. T. C.; b, patient R. H. J.; c, patient C. S. S. (anticentromere pattern); d, patient C. A. C.; e, patient A. G. L.; f, patient L. S. Z. ; g, pooled IgG.

non-conservative amino acid exchanges: Ser31→Asn, Asn52-→Asp and Ser59→Asn, whereas the other five nucleotide substitutions in framework region 3 (FR3) resulted in three amino acid replacements: Gln81→Glu, Ala84→Val and Ala93→Thr (Fig. 5). The D segment of mAbs B-24 and B-33 displayed the highest homology with the germline DXP′1 gene segment.[34] The J_H segment was derived from the J_H6b germline gene.[35]

L chains of B-24 and B-33 were 98% homologous to V_λIII.1 (also known as DPL23), a germline V_λIII gene segment.[36] They

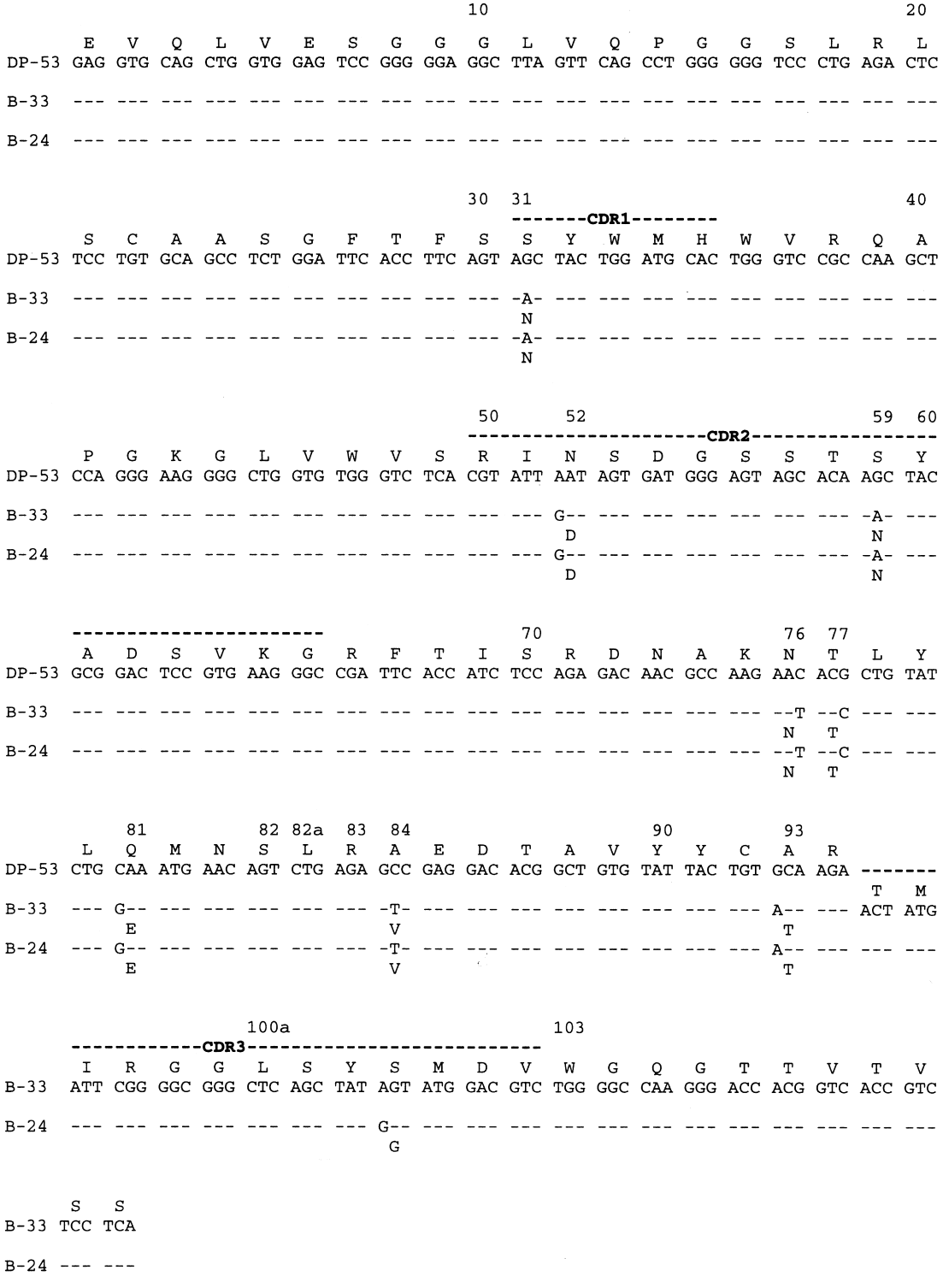

Figure 5. Nucleotide sequence of the V_H3 segments of monoclonal antibody (mAb) B-33 and B-24 compared with that of germline gene DP-53. The deduced amino acid sequence is shown above. A dash indicates identity to the DP-53 nucleotide sequence. Amino acid numbering and H-CDR3 are as defined by Kabat *et al.*[51]

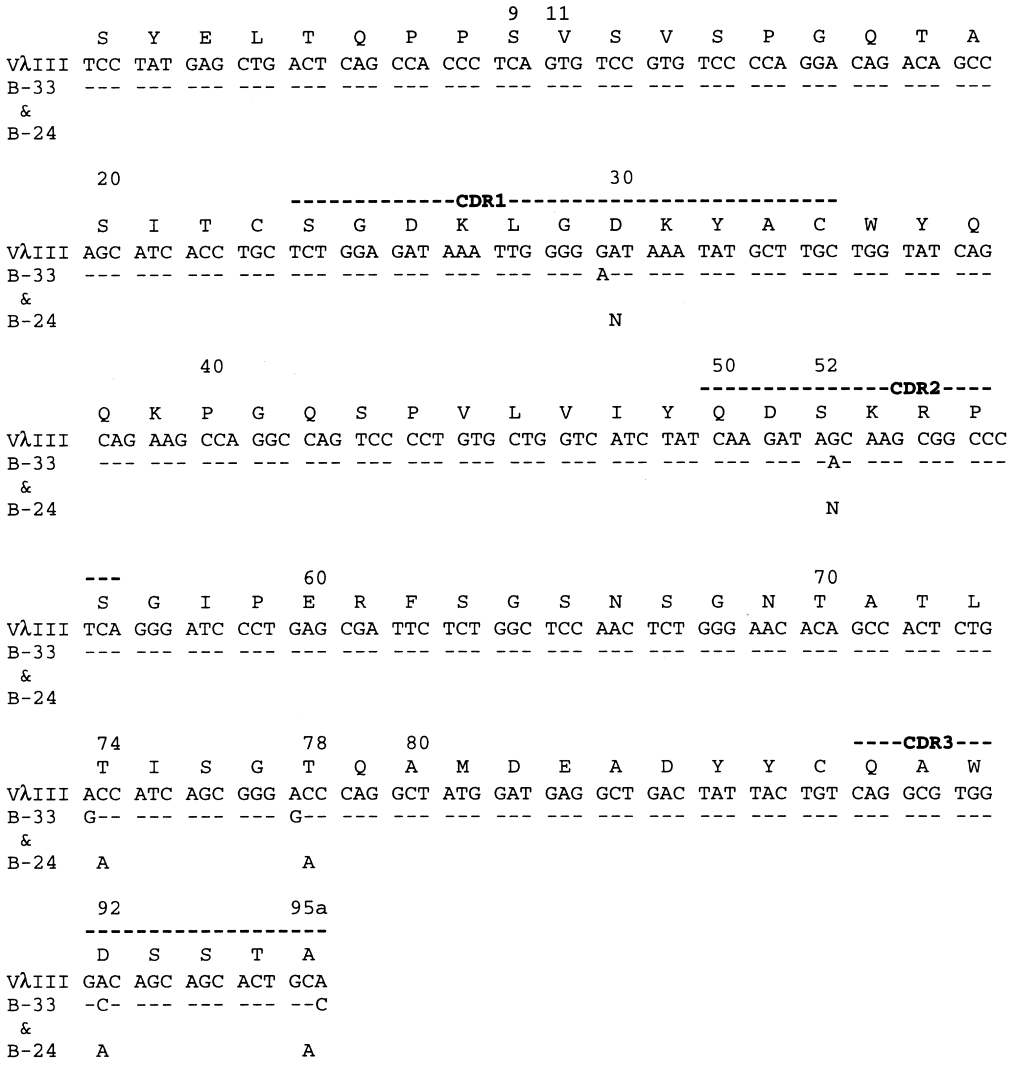

Figure 6. Nucleotide sequence of the V_H3 segments of monoclonal antibodies (mAbs) B-33 and B-24 compared with that of germline gene V_λIII.1. The deduced amino acid sequence is shown above. A dash indicates identity to the V_λIII.1 nucleotide sequence.

differed in six nucleotide substitutions, one of which was silent. The other five resulted in non-conservative amino acid replacements – three of them were located in the CDR: Asp30→Asn, Ser52→Asn and Asp92→Ala, whereas the other two were in FR3: Thr74→Ala and Thr78→Ala. (Fig. 6). The J_λ segment was an unmutated copy of the germline $J_\lambda1$ segment.[37] All of these sequences are available from the EMBL/GenBank under the accession numbers: U76673–5.

The H-CDR3 of human mAbs B-24 and B-33 encodes 13 amino acid residues. Five of these amino acid residues were found to be encoded by the DXP′1 gene (in the third possible reading frame), five by the J_H6b segment and three by the junctional (N) sequences between the D–J_H elements. Of the two point mutations found in the copy of the DXP′1 gene used by these mAbs, a replacement of Val97 by Ile was found in only one. It is important to point out that the H-CDR3 of mAbs B-33 and B-24 were found to be identical in length and amino acid sequence, with the exception of a single non-conservative replacement of Gly100D by Ser, generated by somatic mutation, in mAb B-33 (Fig. 7).

DISCUSSION

AutoAbs directed against a wide spectrum of intracellular molecules are typically found in the serum of patients with autoimmune diseases. Some of these intracellular molecules are well-known autoAgs, but others are still to be identified. In this work, we report the identification of a novel target of autoAbs (the CI-MPR/IGF-2R, found preferentially in the late endosomal compartment) by mAbs raised from a patient with scleroderma as well as by five different serum samples obtained from individuals with different pathological conditions.

Focusing on characterization of the unidentified structure targeted by these autoAbs, we observed that the immunofluorescence staining pattern of the human mAbs under investigation showed large differences among single Hep-2 cells, probably as a result of the relationship between cell-surface expression of receptor molecules, the state of differentiation of particular cells and the phenotypic properties of a single cell. To identify a labelled structure in the complex organization of the membrane system, establishing a

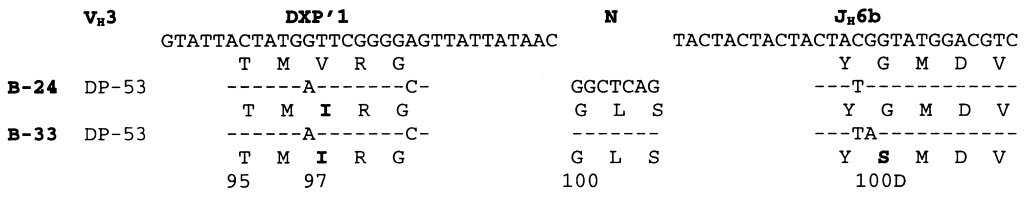

Figure 7. H-CDR3 sequences of monoclonal antibodies (mAbs) B-33 and B-24. There is an addition of seven nucleotides between the D and J segments, originated in the VDR recombination event. Only the portion of the J_H segment that forms the H-CDR3 is represented.

relationship between a morphological description and an antigenic definition of the individual components of a given compartment is essential. The vesicles recognized by human mAb B-33 were also stained by anti-CI-MPR Ab, showed a weak reactivity with anti-LAMP Ab and were negative for Abs to clathrin or transferrin receptor, being morphologically similar to endosomal structures. Immunolocalization studies have revealed that at steady state, most of the CI-MPR is located in the late endosomal compartment, the rest being distributed over the plasma membrane, early endosomes and the trans-Golgi network, with very low or undetectable amounts in structures identified as lysosomes.[8,9,38]

Fluorescence microscopy and video recording of live cells to trace the passage of ligand–receptor complexes have identified the endosomal compartment as an extensive network of tubular cisternae. Endocytosed material entering this reticulum shows discrete swellings, identified by electron microscopy as multivesicular bodies, which move along the reticulum towards the pericentriolar area.[39] This means that the endocytic organelles are highly dynamic and their structure is continuously remodelled. Obviously, these dynamic features complicate the interpretation of currently static morphological images obtained in the laboratory.

The IGF-2R has been found on several types of lymphoid cells. These include PHA- and anti-CD3-activated T cells,[40,41] as well as alveolar macrophages and freshly isolated peripheral blood monocytes.[42] Autocrine or paracrine effects of IGF-2 in T-cell proliferation, differentiation and function have been suggested via the IGF-2R.[40,41,43] Although the CI-MPR/IGF-2R has never been associated with any autoimmune manifestation, there is still much to be learned about its precise function as a target of autoAbs. Thus, a key question remains to be answered: are these Abs pathogenic? Many intracellular Ag are involved in essential cell functions; when reacting with the specific autoAb this situation could lead to cessation of essential activities,[1] so CI-MPR Abs could directly interfere with sorting of lysosomal enzymes or affect signal transduction by simulating binding of ligands at the cell surface, as does IGF-2. On the other hand, the fact that circulating anti-CI-MPR/IGF-2R were detected in serum samples of certain individuals with autoimmune manifestations indicates that the autoreactivity profile of the mAb generated was not an exceptional event, but rather the imprint of a certain autoimmune condition, although it was not possible to correlate the presence of anti-CI-MPR/IGF-2R Ab with a defined autoimmune disease. More work will be required to link this specificity to actual pathology.

EBV transformation has been used to immortalize the B-cell repertoire in normal subjects as well as in patients with diverse autoimmune diseases.[3,4,11,14,15] Kozbor *et al.* reported that employment of EBV transformation prior to cell fusion could be useful for rescuing low-frequency autoreactive B-cell precursors,[44] and thus be of interest in the generation of human monoclonal autoAbs with specificities poorly represented in serum. As the reactivities of the mAbs obtained from our patient were not consistent with those observed in her serum, it could be argued that the method chosen for the production of human mAbs favoured the selection of lymphocyte subsets producing irrelevant antibody reactivities to those found in the serum. Furthermore, it has been reported that EBV infects CD21-bearing B lymphocytes, a receptor present in resting B cells but not in proliferating B cells.[45] Similarly, Ab-producing cells in the terminal differentiation stage lose surface CD21. Lack of EBV binding to cells in S-phase or to plasma cells could explain the lower rate of EBV-induced immortalization observed in cells spontaneously producing Ab *in vivo*, as is occasionally the case for cells obtained from subjects with active-phase autoimmune diseases.

The results presented here indicate that several clones of heterohybridomas (B-24) reacting against this novel autoAg showed multispecific properties similar to those found in natural autoAb. Characteristically, natural autoAb are polyreactive, can recognize a variety of self- and exogenous molecules, and bind to a wide range of Ags with intrinsic low affinity. A feature commonly found among this kind of Ab is that it is encoded by germline genes with no, or only a few, mutations.[46] Another line of research has arisen from the comparison of the nucleotide sequences of mAbs B-24 and B-33, which indicates that clones from which they derive are closely related. The search for common characteristics at the amino acid level that could explain the recognition of the same structure, as well as the different behavior of these mAbs, showed that the V-region of the H and L chains of mAbs B-33 and B-24 were identical, except for one residue located in the H-CDR3 of mAb B-33.

The important role played by the H-CDR3 in Ab specificity, as suggested by Chothia *et al.*,[47] may arise from the central position of this structural element in the binding site rather than from its size. In fact, the H-CDR3 structures described for many human polyreactive Abs are highly divergent in length and composition, and do not allow the identification of any obvious common motif possibly responsible for polyreactivity.[48] On the other hand, it has been established that point mutations, especially in the H-CDR3, greatly alter Ab reactivity. Although limited to a single Ab pair, the present findings support previous evidence suggesting that both monoreactive and polyreactive Abs utilize identical V_H and V_L gene segments in conjunction with discrete primary

structures of the H-CDR3.[48] These data provide additional evidence on how a single amino acid substitution in the H-CDR3 of an Ab can profoundly affect the binding capacity for self-Ag, thus indicating that polyreactivity is a very fragile property and is dependent upon the primary structure of the H-CDR3.[49,50]

The results obtained suggest that the role of polyreactive IgM extends far beyond that of providing the structural elements of the idiotypic regulatory network, and that of providing the first line of defence in the early phases of infection, supporting the notion that B cells producing autoantibodies may arise through an initial selection for both autoreactivity and polyreactivity. Such cells, in turn, may be precursors of cells that make Ab of higher affinity and narrower selectivity after mutation and selection by autoAg, which finally drives formation of IgG autoAb. These features can only be explained by the presence of cognate T-cell help, which implies that T cells participate in, and regulate, the autoimmune response. Thus, the autoimmune response to CI-MPR/IGF-2R is driven by this autoAg and involves both T- and B-cell recognition, identical to the immune responses to exogenous Ag.

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