A Novel Mannose 6-phosphate Specific Antibody Fragment for Diagnosis of Mucolipidosis type II and III

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Sandra Pohl, Thomas Braulke, and Sven Müller-Loennies

13.1 Introduction

Eukaryotic cells of animals have developed a specialized organelle for the degradation and recycling of macromolecules, called the lysosome (De Duve 1963). The breakdown of these macromolecules is carried out by more than 60 acid hydrolases such as proteases, nucleases, glycosidases, phosphatases, lipases etc. (Luzio et al. 2007). Newly synthesized lysosomal hydrolases are equipped with mannose 6-phosphate (Man6*P*) residues on high-mannose type *N*-glycans. This marker is generated in the Golgi apparatus in a two-step enzymatic process in which first a *N*-acetylglucosamine 1-phosphate (GlcNAc1*P*) residue is transferred to a terminal mannose (Man) residue. In a second step the enzymatic hydrolysis of the GlcNAc uncovers the Man6*P* residue. Man6*P* functions as recognition marker for specific receptors required for lysosomal targeting of acid hydrolases. Importantly, from a medical point of view, also extracellular Man6*P*-containing proteins can be internalized and transported to the lysosomes via Man6*P*-receptors which are located also at the plasma membrane (Kornfeld and Mellman 1989; Braulke and Bonifacino 2009). Whereas the majority of the over 50 known lysosomal storage disorders are caused by inherited defects of single lysosomal enzymes or lysosomal membrane proteins, the failure to generate Man6*P* leads to a deficiency of multiple enzymes resulting in mucolipidosis (ML) type II and type III (Futerman and van Meer 2004).

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The diagnosis of MLII and III is only carried out in a few specialized laboratories throughout the world and is based on the biochemical and genetic analysis of biomaterial isolated from patients. Diagnosis of MLII and III would greatly benefit from the development of confirmatory assays easier to perform also in routine analytic laboratories on suspicious patients. For this purpose the specific detection of Man6*P* in glycoproteins by an antibody would be ideal.

This application in mind, we have immunized a rabbit with a neoglycoconjugate of a mixture of oligosaccharides from yeast containing Man6*P* residues and bovine serum albumin (BSA) known to induce a polyclonal anti-Man6*P* response (Braulke et al. 1987; Braulke et al. 1988). The antigen binding domain of an antibody can be expressed in *Escherichia coli* as a single-chain antibody fragment (scFv) in which the VH and VL domains of the antibody are joined by a flexible polypeptide linker (Bird et al. 1988; Huston et al. 1988) and genetically fused to surface proteins of, e.g., filamentous phage. The surface display then allows selection and amplification, a technique called "Phage Display" [for further information see (Barbas et al. 2001)]. Since the generation of monoclonal antibodies (mAb) from rabbits is far from being routine due to the lack of appropriate fusion cell lines, we have referred to phage display technology to generate a scFv specifically binding to Man6*P* (Müller-Loennies et al. 2010).

In this review we describe the genetic and biochemical background of mucolipidoses and the development of a novel antibody scFv (scFv M6P-1) for the

- Easy diagnosis of MLII and III by western blots.
- Selective purification of recombinant high-affinity uptake forms of lysosomal enzymes on an affinity matrix.
- Immunohistological staining of lysosomes.

Furthermore, scFv M6P-1 can be applied to the quantitation of Man6*P* in glycoproteins, the isolation of the Man6*P*-proteome of cells and organs and has aided in the investigation of alternative protein trafficking pathways to the lysosome.

13.1.1 Generation of the Man6*P* Recognition Marker

Soluble lysosomal enzymes and secretory proteins are synthesized in the endoplasmic reticulum (ER). The *N*-terminal signal sequence directs their translocation into the ER lumen and is subsequently cleaved off by the signal peptidase. For *N*-glycosylation a preformed oligosaccharide core composed of three glucoses (Glc), nine Man and two GlcNAc, ($Glc_3Man_9GlcNAc_2$) is transferred to selected asparagine residues on the nascent protein prior to protein folding [Fig. 13.1, (Rothman et al. 1978; Ruddock and Molinari 2006)]. The transferred core oligosaccharides are then subject to 'trimming' reactions initiated by glucosidase I in the ER before completion of translation (Kornfeld and Kornfeld 1985). The two other glucose residues are subsequently hydrolyzed by glucosidase II in the ER. The monoglucosylated core glycan intermediate is recognized by the lectins calnexin and calreticulin which function as molecular chaperones until the protein is properly folded

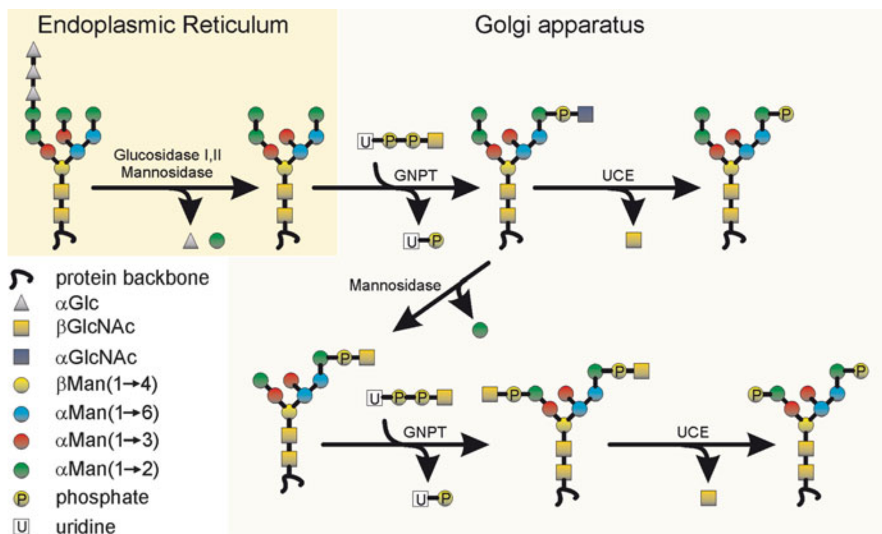

Fig. 13.1 Schematic drawing of the enzymatic formation of Man6*P* residues on *N*-glycans of lysosomal proteins. The generation of these residues is catalyzed in a two-step reaction initiated by the transfer of UDP-GlcNAc to selected C6 hydroxyl groups of mannoses in a *cis*-Golgi compartment by UDP-*N*-acetylglucosamine (UDP-GlcNAc):lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GNPT, GlcNAc-1-phosphotransferase; EC 2.7.8.17) followed by hydrolysis of the GlcNAc residue in the *trans*-Golgi apparatus by the *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase (UCE, 'uncovering enzyme'; EC 3.1.4.45). Some of the *N*-glycan chains are equipped with a second phosphodiester group. Phosphorylation of mannose residues in the α(1→6)-branch occurs before the trimming of mannoses in the α(1→3)-branch by α-mannosidase I. Therefore, phosphorylated glycans may contain 5–7 mannose residues. Depending on the lysosomal enzyme and the cell type, certain oligosaccharides are converted into hybrid or complex type sugar chains (not shown). Therefore, monophosphorylated oligosaccharides are also found in the α(1→6)-branch of hybrid-type oligosaccharides. The subcellular compartments in which the reactions take place are indicated as boxes

(Parodi 2000). The removal of the third glucose residue releases the folded glycoprotein from the chaperones and allows the attack of α-mannosidase I for further trimming reactions in the ER resulting in the formation of octamannosyl high-mannose type chains.

An important modification of *N*-glycans on lysosomal enzymes is the formation of Man6*P* residues distinguishing these glycoproteins from other classes of newly synthesized glycoproteins. Two enzymes catalyze the generation of Man6*P* residues: UDP-*N*-acetylglucosamine (UDP-GlcNAc):lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase; EC 2.7.8.17) and the *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase ('uncovering enzyme', UCE; EC 3.1.4.45).

The initial transfer of GlcNAc1*P* from UDP-GlcNAc to selected C6 hydroxyl groups of mannoses occurs in a *cis*-Golgi compartment that results in a phosphodiester intermediate. The hydrolysis of the covering GlcNAc residue from the phosphodiester

by the UCE in the *trans*-Golgi apparatus exposes the Man6*P* marker (Fig. 13.1). Within the Golgi apparatus, some of the monophosphorylated oligosaccharide chains are equipped with a second phosphodiester group. Phosphorylation of mannose residues in the α(1→6)-branch occurs before the trimming of mannoses in the α(1→3)-branch by α-mannosidase I, resulting in high-mannose type oligosaccharides containing 5–7 mannose residues (Fig. 13.1). Depending on the lysosomal enzyme and the cell type, different numbers of oligosaccharides can be converted into hybrid or complex type sugar chains by the transfer of GlcNAc, galactose (Gal), fucose (Fuc), or *N*-acetylneuraminic acid (Neu5Ac) residues. Therefore, monophosphorylated oligosaccharides are also found in the α(1→6)-branch of hybrid-type oligosaccharides (Varki and Kornfeld 1983; Goldberg and Kornfeld 1983; Lazzarino and Gabel 1989).

13.1.2 Mucolipidosis Type II and III

The GlcNAc-1-phosphotransferase complex is composed of three subunits ($\alpha_2\beta_2\gamma_2$) that are encoded by two genes, *GNPTAB* and *GNPTG* (Kollmann et al. 2010). The GlcNAc-1-phosphotransferase activity is lacking or reduced in two distinct autosomal recessive human diseases impairing lysosomal enzyme trafficking and lysosomal function, mucolipidosis (ML) II and MLIII, respectively. Although rare diseases, they have been the subject of extensive studies in the last 40 years since their first description. These studies were crucial for the discovery of the Man6*P*-dependent lysosomal enzyme trafficking pathway [reviewed in (Kornfeld and Sly 2001; Kollmann et al. 2010)].

Patients diagnosed with MLII alpha/beta or MLIII alpha/beta (formerly known as I-cell disease or MLII and MLIIIA, respectively) are either homozygotes or compound heterozygotes for mutations in the *GNPTAB* gene localized on chromosome 12q23.3 (Kudo et al. 2005; Tiede et al. 2005b). The MLIII gamma patients (formerly known as pseudo-Hurler polydystrophy or MLIIIC) are homozygotes or compound heterozygotes for mutations in the *GNPTG* gene localized on chromosome 16p13.3 (Raas-Rothschild et al. 2000). The revised nomenclature of mucolipidoses is based on the clinical phenotype and the disease-causing gene defect and is important for clinicians and patients, as *GNPTG* mutations cause a milder phenotype and have better prognosis than do *GNPTAB* mutations (Cathey et al. 2008). Until now approximately 100 mutations in the *GNPTAB* gene have been described whereas 20 different mutations were reported in the *GNPTG* gene of MLIII gamma patients (Kollmann et al. 2010).

Clinical symptoms of MLII patients are characterized by dwarfism, skeletal abnormalities, facial dysmorphism, stiff skin, delayed development, mental retardation and cardiomegaly leading to death between 5 and 8 years of age. In MLIII gamma patients a later onset of clinical symptoms and a more slowly progressive course is observed allowing survival into the 8th decade. Because of progressive stiffness of hands and shoulders and musculoskeletal changes, MLIII gamma is

often misdiagnosed for a rheumatological disorder (Kelly et al. 1975; Spranger et al. 2002; Cathey et al. 2008).

13.1.3 Current Diagnostic Analysis of MLII and MLIII

The clinical diagnosis of MLII or III can be confirmed by biochemical analyses. The loss or reduced capability to generate Man6*P* leads to a massive secretion of the lysosomal enzymes into the extracellular milieu and the circulation, and an intracellular deficiency of multiple lysosomal enzymes (Kornfeld and Sly 2001). The intracellular deficiency of lysosomal enzymes causes subsequently an accumulation of undegraded material in lysosomes, which is visible by light microscopy as phase-dense inclusion bodies in fibroblasts of affected patients (Leroy and Demars 1967). The activity of several lysosomal enzymes (e. g. β-hexosaminidase, β-glucuronidase, β-galactosidase, α-mannosidase and arylsulfatase A) can be measured in serum, or media and cell extracts of cultured fibroblasts from clinically diagnosed MLII and MLIII patients. In comparison to cells of healthy individuals the activities of lysosomal enzymes are reduced in patient cells but increased in serum and conditioned cell media demonstrating the missorting of lysosomal enzymes (Kornfeld and Sly 2001). During the transport along the biosynthetic pathway in healthy cells many inactive lysosomal precursors undergo proteolytic processing steps leading to mature, active enzymes. The modifications of lysosomal enzymes are dependent on the pH and the intracellular compartment and can be used as an indicator for proper sorting processes (Hasilik and Neufeld 1980). Radioactive pulse-chase experiments in fibroblasts of diagnosed MLII or MLIII patients followed by immunoprecipitation of certain lysosomal enzymes is useful to demonstrate the missorting of lysosomal enzymes (Tiede et al. 2005a; Pohl et al. 2010b). The direct measurement of the GlcNAc-1-phosphotransferase activity in fibroblasts requires the synthesis and purification of metabolically labelled [^{32}P]UDP-GlcNAc (Reitman and Kornfeld 1981). Both methods are cumbersome and therefore cannot be routinely used for diagnosis.

Despite the major progress made during the last decade allowing the identification of the molecular defects in MLII and MLIII by direct sequencing of *GNPTAB* and *GNPTG*, these methods are laborious and expensive, and intronic mutations are not always detectable. Therefore, a rapid, convenient and sensitive method would greatly facilitate the diagnosis of MLII and MLIII.

13.2 Generation and Characterization of scFv M6P-1

13.2.1 Immunization and Selection

Upon mild hydrolysis of *Pichia (Hansenula) holstii* NRRLY-2448 yeast mannan oligosaccharides can be obtained which contain Man6*P*. The structural analysis revealed that the hydrolysate consists of a mixture of oligosaccharides (Fig. 13.2)

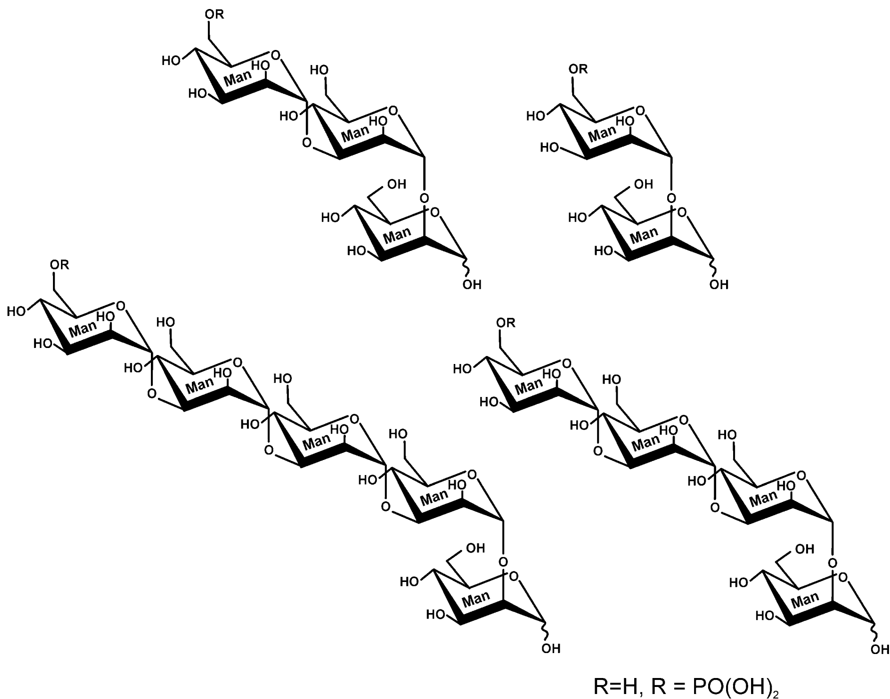

Fig. 13.2 Chemical structures of Man6*P*-containing oligosaccharides from *Pichia (Hansenula) holstii* (Bretthauer et al. 1973; Parolis et al. 1998; Ferro et al. 2002)

and the majority are pentasaccharides (Bretthauer et al. 1973; Parolis et al. 1996; Parolis et al. 1998). Therefore, this preparation is referred to as pentamannose 6-phosphate (PMP). As opposed to *N*-glycans, in which the terminal phosphorylated mannose residues are connected αMan(1→2)αMan, the PMP contains terminal α(1→3)-linked mannoses in which only the reducing end is formed in an α(1→2)-linkage [Fig. 13.2, (Fischer et al. 1980; Parolis et al. 1996; Parolis et al. 1998)]. The conjugation of PMP to BSA and immunization with this conjugate induces a polyclonal antibody response in rabbits which binds Man6*P* also in glycoproteins [PMP-BSA, (Braulke et al. 1987)]. The basis for this observation is an apparent structural similarity of α(1→2)- and α(1→3)-linked Man_2 leading to a cross-reaction with antibodies.

For the generation of a Man6*P*-specific scFv from an immunized rabbit, a library of scFv was assembled by standard genetic procedures (Barbas et al. 2001). This was achieved by taking bone marrow from both legs and spleen, extraction of total RNA, reverse transcription into cDNA and PCR amplification of genes coding for the variable domains of antibody heavy (VH) and light (VL) chains. The amplified genes were then assembled into full length scFv by PCR overlap extension and ligated into the phagemid vector pComb3XSS (Barbas et al. 2001). Transformation of *E. coli* yielded 5.7×10^6 transformants and after induction of phage-production

by superinfection with M13KO7 helper phage, 10^{12} phages were obtained for the selection. The selection and enrichment of phage bound scFv ("panning") specific for Man6*P* was performed on PMP-BSA immobilized on ELISA plates. The selection of carbohydrate antibodies by phage display is difficult to achieve due to often low avidities. Because the conditions to achieve optimum binding were unknown, we have varied the amounts of immobilized PMP-BSA (3–100 pmol/well) used for panning. The outcome of each panning round was analyzed by phage-ELISA and successful enrichment was only seen at 50 pmol immobilized antigen after five rounds of panning. Some binding was also seen after three rounds, which was, however, not specific for Man6*P*.

13.2.2 Expression and Biochemical Characterization

For purification by immobilized metal ion affinity chromatography (IMAC) and detection 5xHis-tag and a c-myc were attached to the *C*-terminus of the scFv M6P-1 protein sequence, respectively. Expression in *E. coli* following the protocol of MacKenzie and To (1998) yielded soluble protein, which was extracted from the periplasm by treatment of harvested cells with the polycationic membrane active peptide Polymyxin B (0.1 mg/ml final conc. in ice cold buffer). The soluble protein could be collected from the supernatants of three extractions by IMAC. Separation of monomers from oligomeric scFv was achieved by gel filtration (Fig. 13.3). The cDNA sequence analysis revealed an unpaired cysteine at the beginning of complementarity determining region (CDR) 2 VH (Fig. 13.4) which is also present in the germline sequence IGHV1S40*01 (Giudicelli et al. 2006). The substitution of this residue by serine (scFv M6P-1S) or alanine improved expression levels in *E. coli* three- to four-fold and increased the protein stability whereas the affinity towards Man6*P* was not affected (Fig. 13.5). After purification to homogeneity and lyophilization in phosphate buffered saline (PBS) pH 7.2 containing 1% PEG 8,000, 5 mM EDTA and 250 mM trehalose (Carpenter et al. 1993; Draber et al. 1995) the protein was stable for over a year without loss of activity.

13.2.3 Analysis of Binding by ELISA

To achieve comparable results in ELISA and ELISA inhibition studies using scFv, in general, freshly prepared solutions of purified mono- or dimer scFv should be used to avoid influences from avidity effects. Although scFv contain only a single antigen binding site, they are prone to form fully functional oligomers (Dolezal et al. 2000) and the ratio at equilibrium depends on the affinity between the VL and VH interfaces and the length of the linker (Glockshuber et al. 1990).

13.2.3.1 ELISA with Soluble scFv M6P-1

For ELISA, the outcome of the experiment largely depends on the amount of immobilized antigen and the concentration of the primary antibody. Due to the

Fig. 13.3 Gelfiltration on Superdex HR75 (GE Healthcare; 30 cm × 1.5 cm in PBS, pH 7.2, 0.5 ml/min) of recombinantly expressed scFv M6P-1S after purifica-tion by affinity chromatography. Two major and one minor fraction can be separated consisting of monomeric (1), dimeric (2) and oligomeric (3) scFv. All fractions are able to bind the antigen

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VL
                                              CDR 1                              CDR 2
                1         10        20        30        40        50        60
IGKV1S12*01     DPVLTQTASPVSAAVGGTVTISCQSSQSVY....NNNWLSWYQQKPGQPPKLLIYGA.......S
scFv M6P-1      ELVMTQTESPVSAAVGGTVTIKCQSSQSV......NNRLAWYQQKPGQRPKLLIYSA.......S

                                                            CDR 3
                  70        80        90        100
IGKV1S12*01     TLASGVP.SRFKGSG..SGTQFTLTISGVQCDDAATYYCQGAYSGNIY
scFv M6P-1      TLASGVP.SRFKGSG..SGTQFTLTISDLEWGDAATYYCHGGYRSNDDRYAFSGGTELEIL

VH
                                              CDR 1                              CDR 2
                1         10        20        30        40        50        60
IGHV1S40*01     Q.SLEESGG.DLVKPGASLTLTCTASGFSFS...SSYYMCWVRQAPGKGLEWIACIYAGS.SGST
scFv M6P-1      Q.SVKESEG.DLVKPGASLTLTCKASGFDFT....WYTMNWVRQAPGKGLEWIACIGAGV.YGSN

                                                            CDR 3
                  70        80        90        100
IGHV1S40*01     YYASWAK.GRFTISKTSS.TTVTLQMTSLTAADTATYFCAR
scFv M6P-1      YYASWAK.GRFTISKASS.TTVTLQMTSLTVADTATYFCARDGINGGYDIWGPGTLVTVSS
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Fig. 13.4 Primary structure of the scFv M6P-1 deduced from its cDNA sequence. The CDR of heavy and light chains are indicated and alignment to its germline precursor assigned according to Giudicelli (2006)

monovalent nature of scFv and the often low affinities of anti-carbohydrate antibodies, the ELISA is even more affected by these factors. Thus, when using neoglycoconjugates made with isolated oligosaccharides a high ligand to protein

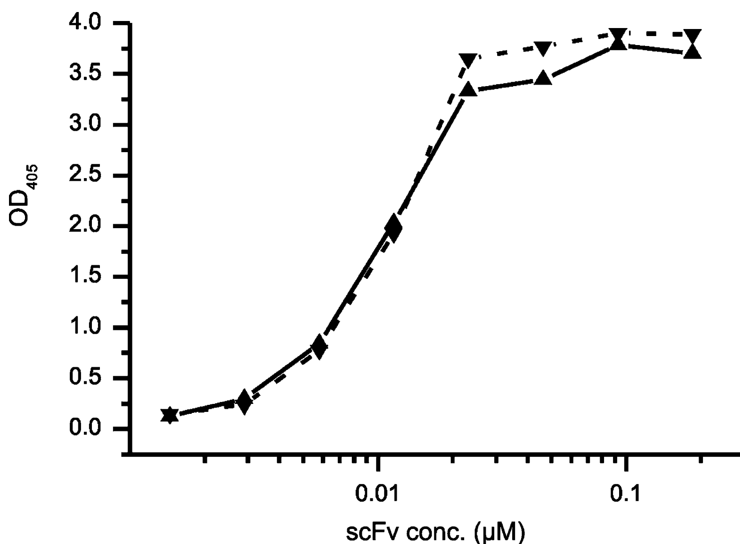

Fig. 13.5 ELISA binding assay of recombinant scFv M6P-1 (*broken line*, *triangle down*) and scFv M6P-1S (*solid line*, *triangle up*) against immobilized PMP-BSA (45 pmol/cup). The scFv starting concentration was 0.18 μM (5 μg/ml)

ratio would be desirable. However, neoglycoconjugates with a high ligand to protein ratio not always give the best results in ELISA binding assays when native BSA is used (own observation). It may thus be advantageous to use denatured and reduced BSA for the generation of neoglycoconjugates (Houen and Jensen 1995) for this purpose.

In any case, to determine the ligand concentration suitable for ELISA the binding reactivity should be tested in an ELISA checkerboard titration in which binding at different scFv starting concentrations is measured against varying concentrations of immobilized ligand. For ELISA using scFv M6P-1 (Fig. 13.5) we have used PMP-BSA (molar ratio of PMP:BSA of 20:1) containing 3–100 pmol of ligand with 45 pmol giving the best result when purified scFv was added at a starting concentration of 5 μg/ml. Bound scFv was detected by incubation with the anti-c-myc mAb 9E10 (gift from Dr. C. R. MacKenzie, NRC, Ottawa, Canada), a HRP-conjugated goat-anti-mouse IgG H + L (Dianova, Hamburg, Germany) and diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (AzBTS-$(NH_4)_2$, Sigma-Aldrich) as substrate.

13.2.3.2 ELISA Inhibition

Inhibition of binding in ELISA is performed conveniently by first titrating the inhibitor in buffer in a separate non-treated polystyrene plate (e.g., Nunc V96 MicroWell) starting in the first row at double concentration of the highest inhibitory concentration to be tested in a volume of, e.g., 30 μl. The same volume of antibody is then added at a constant concentration which yields an OD within the linear range

of the colour reaction and which is sufficiently strong to allow sampling of the inhibition (OD 1.0–2.0). For best results quadruplicate measurements at each inhibitor concentration should be performed and titrations starting at a concentration sufficiently high to achieve complete inhibition over a few titrations and ending with several dilute concentrations of inhibitor at which no inhibition is seen. After preincubation (1 h, 37°C) 50 μl are then transferred to a plate coated with ligand and developed like a regular ELISA. The ELISA inhibition data should then be analyzed by plotting the OD versus the inhibitor concentration and applying an error-weighted non-linear logistic fitting function as implemented in statistical analysis software.

For ELISA inhibition with scFv M6P-1 (Fig. 13.6) we have used 0.6 μg/ml final concentration of scFv and inhibitor concentrations ranging from 0.01 to 100 mM. Binding was measured against 45 pmol PMP-BSA/well. The amount of bound antibody was then determined as for normal ELISA. Using one plate per inhibitor, data points can be measured in quadruplicates over 22 concentrations spanning six orders of magnitude which is sufficient to sample the whole inhibition curve and determine the concentration yielding 50% inhibition (IC_{50}) by fitting to a logistic function. Such an analysis for scFv M6P-1 is shown in Fig. 13.6 showing the strong

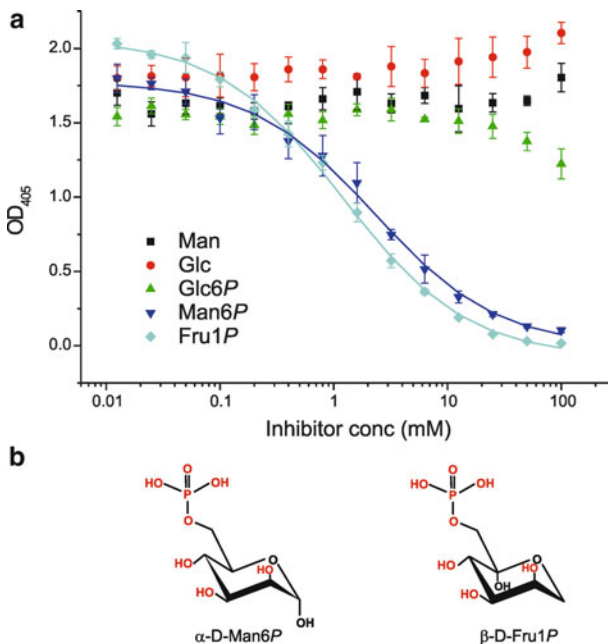

Fig. 13.6 ELISA inhibition data using scFv M6P-1 as primary antibody, PMP-BSA as immobilized antigen and Man6*P*, Glc6*P*, Fru1*P*, Man, and Glc as inhibitors (**a**) at the indicated concentrations. Experimental details are described in Müller-Loennies et al. (2010). Structural comparison of Man6*P* and Fru1*P* (**b**). Fig. (**a**) from Müller-Loennies et al. (2010), with permission

inhibition by Man6*P* and fructose 1-phosphate (Fru1*P*) and no inhibition by Man, Glc and Glc6*P*.

The observed cross-reaction of Man6*P* and Fru1*P* can be explained by the structural similarity due to the same relative orientation of hydroxyl groups at C2-C3-C4, and C3-C4-C5, respectively, with a similar position of the phosphate. Thus, the discrimination between Glc6*P* and Man6*P* is likely due to an unfavourable equatorial position of the C2 OH for binding.

13.2.4 Analysis of Binding by ITC-Microcalorimetry

The comparison of ELISA IC_{50} values allows the ranking of different ligands according to their relative affinities; however, the absolute affinities in terms of K_d cannot be obtained. Therefore we have performed isothermal titration microcalorimetry (ITC) measurements. Using 10 mM of Man6*P* or Fru1*P* as ligands and purified dimeric scFv M6P-1 (11 mg/ml in 100 mM PBS, pH 7.2 containing 150 mM NaCl) we have performed ITC experiments using an ITC200 calorimeter (Microcal Inc., Northampton, MA, USA). As an example, the measurement of scFv M6P-1S against Fru1*P* is shown in Fig. 13.7. For this experiment the measurement cell of the calorimeter was filled with the antibody solution and the ligand loaded into the injection syringe from where it was injected into the cell in 2 μl portions. Twenty injections were performed with 3 min equilibration times between injections and the evolved heat measured with the first injection not considered for data analysis. The heat of dilution was measured for the same number of buffer injections which was subtracted from the sample data. The K_d values of Man6*P* and Fru1*P* were 30 μM for the germline derived scFv sequence (Müller-Loennies et al. 2010) and for the VH Cys/Ser mutant (Fig. 13.7).

13.2.5 Western Blot and Immunoprecipitation

Western blot analysis against the purified lysosomal enzyme arylsulfatase B (kindly provided by M. Vellard, Biomarin, Navato, USA) immobilized on a nitrocellulose membrane at concentrations ranging from 1 to 10 ng revealed that scFv M6P-1 can be used for the detection of lysosomal enzymes with a detection limit of a few ng, depending on the degree of phosphorylation (Fig. 13.8). For comparison the PMP-BSA glycoconjugate used for the immunization and in ELISA binding assays is shown. This is of particular importance for the commercial production of recombinant proteins for enzyme replacement therapy (ERT) of lysosomal storage diseases. By Western blot analysis the expression levels of bioactive enzyme which contains the essential Man6*P* modification can be assessed easily, allowing the selection of clones producing high levels of recombinant protein and therefore leading to a considerable reduction of production costs. A comparison of methods for the quantitative analysis of Man6*P* in glycoproteins revealed that the degree of phosphorylation maybe underestimated in Western blots (Schröder et al. 2010), possibly due to steric hindrance.

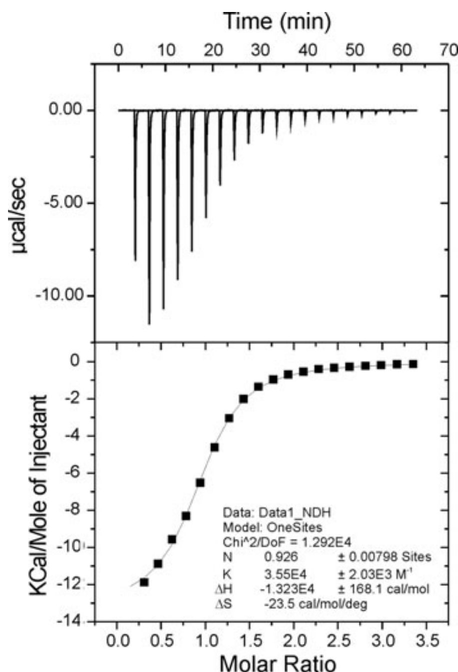

Fig. 13.7 Isothermal titration microcalorimetry (ITC) measurement using scFv M6P-1 in the cell and Fru1*P* as ligand in the injection syringe. The measurement was performed in 100 mM phosphate buffered saline (150 mM NaCl) at pH 7.2. The cell was filled with the antibody solution (11 mg/ml = 0.4 mM) and the ligand (6 mM) loaded into the injection syringe from where it was injected into the cell in 2 μl portions. Twenty injections were performed with 3 min equilibration times between injections and the evolved heat measured with the first injection not considered for data analysis. The data were then subjected to non-linear least squares curve fitting (1 set of binding sites) using MicroCal Origin v. 7.0 analysis software

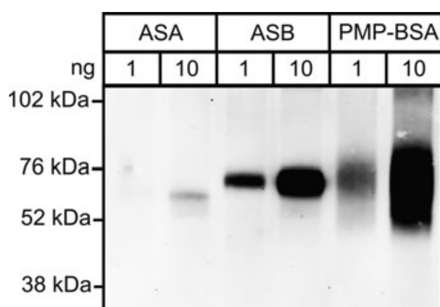

Fig. 13.8 Sensitive detection of Man6*P*-modified lysosomal enzymes. Recombinant lysosomal enzymes arylsulfatase A (ASA), arylsulfatase A (ASB) and a glycoconjugate of pentamannose-6-phosphate and BSA (PMP-BSA) (1 and 10 ng) were separated by SDS-PAGE, blotted onto nitrocellulose and analysed by scFv M6P-1 Western blotting. The positions of the molecular mass marker proteins in kDa are indicated. ASB was kindly provided by M. Vellard, Biomarin, Nevato, CA

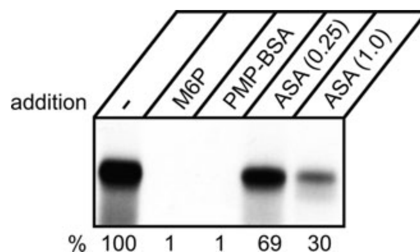

Fig. 13.9 Man6*P*-dependent precipitation of [^{125}I]-labelled ASA after incubation with scFv M6P-1-coupled beads in the absence (100%) or presence of the inhibitors Man6*P*, PMP-BSA and arylsulfatase A (ASA). Bound material was eluted, separated by SDS-P AGE, and visualized by autoradiography

As a first step towards the generation of an affinity support for large scale chromatography we have first tested whether it would be possible, in principle, to couple the scFv M6P-1 to beads without its inactivation. ScFv M6P-1 coupled beads allowed the subsequent immunoprecipitation of Man6*P*-containing glycoproteins from conditioned cell culture media. When a solution (0.5 ml) of iodinated arylsulfatase A (ASA) was incubated for 10 h at 4°C with 50 µl of scFv M6P-1S-conjugated Affi-gel 10 beads (BioRad), the ASA could be precipitated with the beads shown by SDS-PAGE and subsequent visualization by autoradiography (Fig. 13.9). Addition of 5 mM Man6*P* to the reaction abrogated the binding indicating that the reaction was Man6*P* specific. Evaluation of the intensity of the [^{125}I]-labelled polypeptide bands by densitometry or, alternatively, after excision from the dried gel and γ-counting revealed that 18% of the input was precipitated.

Similar results were obtained when conditioned serum-free media from BHK cells which stably overexpressed human ASA (hASA) or transiently expressed mouse cathepsin D (mCtsD) were used (Müller-Loennies et al. 2010). After mixing aliquots of media (0.25 ml, 1:1 ratio) with medium from non-transfected cells or from the other overexpressing cell line, a Man6*P*-dependent precipitation with 50 µl of scFv M6P-1 coupled beads could be shown by Western blotting using anti-hASA and anti-mCtsD polyclonal antibodies (Fig. 13.10).

13.2.6 Immunofluorescence Microscopy and Immunohistochemistry

The scFv M6P-1 antibody can be used as lysosomal marker colocalizing with lysosomal enzymes such as cathepsin D as showed by double immunofluorescence microscopy (Fig. 13.11a). In cerebellar sections of mouse brain cells Man6*P*-containg proteins are detectable by immunohistochemistry (Fig. 13.11b). The stainings were performed in COS7 cells derived from kidney cells of the African green monkey and mouse tissue demonstrating the species-independence of the antibody.

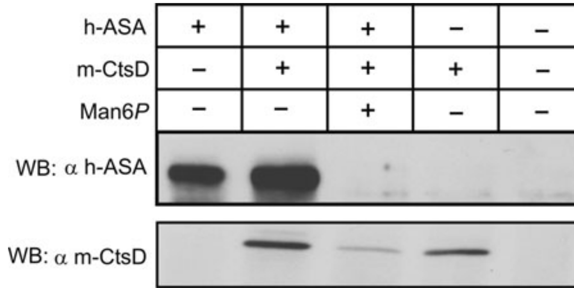

Fig. 13.10 Precipitation of Man6*P*-containing lysosomal enzymes. Purification of Man6*P*-containing lysosomal enzymes from media of overexpressing cells. Serum-free media from BHK cells stably overexpressing human ASA or transiently expressing mouse cathepsin D (mCtsD) were conditioned for 24 h. Aliquots of the media (0.25 ml) were either mixed with 0.25 ml of the other overexpressing cell line or of non-transfected cells and incubated with 50 µl of scFv M6P-1–coupled beads in the presence or absence of Man6*P* (5 mM) for 10 h at 4°C. Then the samples were centrifuged and the supernatant removed. The proteins bound to the scFv M6P-1–coupled beads were solubilized and analyzed by Western blotting using anti-ASA and anti-mCtsD polyclonal antibodies (From Müller-Loennies (2010))

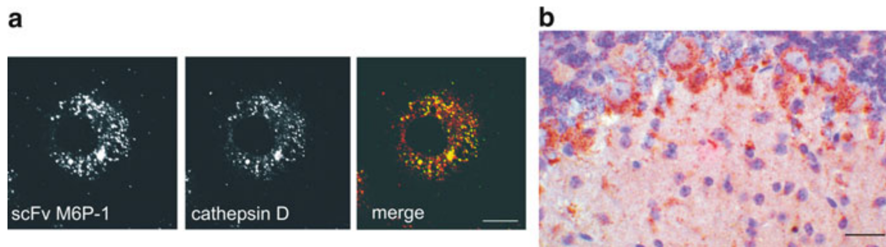

Fig. 13.11 For double immunofluorescence microscopy COS7 cells were fixed, permeabilized and incubated with myc-tagged scFv M6P-1 and the lysosomal marker protein cathepsin D (**a**). The merged picture reveals overlapping distribution (*yellow*). Scale bar, 80 µm. For immunohistochemistry (**b**) paraffin-fixed cerebellar sections of an adult mouse were incubated with myc-tagged scFv M6P-1 and HRP-coupled anti-myc antibodies followed by the peroxidase-diaminobenzidine reaction. The scFv M6P-1 immunostaining is strong in cerebellar Purkinje cells. Scale bar, 80 µm

13.2.7 Diagnostic Application of scFv M6P-1

The high specificity of the scFv M6P-1 antibody for Man6*P* and its ability to recognize these residues on lysosomal enzymes allows its diagnostic application. In MLII and MLIII patients the activity of the GlcNAc-1-phosphotransferase is absent or reduced by mutations in *GNPTAB* or *GNPTG*, respectively. The antibody scFv M6P-1 permits the indirect determination of GlcNAc-1-phosphotransferase activity by western blotting. In detail, cultured fibroblasts obtained from controls and patients clinically diagnosed for MLIII were lysed and cell extracts were separated by SDS-PAGE followed by Western blot analysis. Several Man6*P*-containing

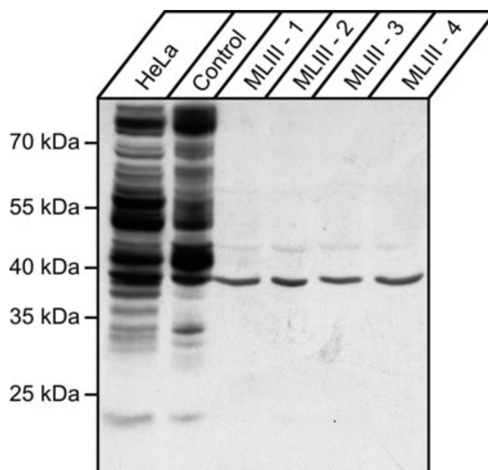

Fig. 13.12 Protein extracts of HeLa cells, and fibroblasts from a healthy control and from four MLIII patients were separated by SDS-PAGE, blotted onto nitrocellulose and analyzed by scFv M6P-1 western blotting. The positions of the molecular mass markers are indicated

proteins in the range between 20 and 100 kDa were detected with different intensities in extracts of human fibroblasts from healthy controls or in HeLa-cells (Fig. 13.12). Man6*P* containing proteins are also detectable in conditioned media of cultured fibroblasts representing secreted lysosomal enzymes (Pohl et al. 2010a). By contrast, extracts of four MLIII gamma patients exhibiting different mutations in the *GNPTG* gene (Persichetti et al. 2009) contained only one prominent Man6*P*-containing protein. The experiment showed that the method is a rapid tool for diagnosis which is less expensive than sequencing of *GNPTAB* and *GNPTG* genes.

13.3 Conclusion and Outlook

The posttranslational modification with Man6*P* is essential for the viability of eukaryotic cells and the generation of functional lysosomes. As such, inherited genetic defects in the biosynthesis of Man6*P* derivatized glycoproteins lead to severe diseases called mucolipidosis. The diagnosis of these diseases has been technically difficult to achieve and could be only carried out in specialized laboratories.

From an immunized rabbit we have isolated by a genetic approach and phage display a novel scFv specifically binding to Man6*P* residues on glycoproteins. Our experiments have shown that the scFv M6P-1 and its derivative M6P-1S can be used for the diagnosis of MLII and MLIII by simple western blotting. Furthermore, the scFv M6P-1S can be used for immunohistological stainings and immunofluorescence microscopy.

Enzyme replacement therapy (ERT) is possible for eight lysosomal storage diseases at present, with recombinant enzymes which have to be expressed in eukaryotic cells and have to be equipped with Man6*P* for cellular uptake with the

exception of β-glucocerebrosidase (Keutzer and Yee 2008). The purification of enzymes for ERT requires expensive chromatographic procedures which do not discriminate between therapeutically active and inactive forms, i.e., with or without Man6*P* modification. Since usually only less than 20% of recombinant proteins when expressed in eukaryotic cells are equipped with Man6*P*, and only a fraction contains high level phosphorylation at more than one site, it would also be desirable to enrich such enzymes because it can be anticipated that such preparations yield enzymes binding with higher affinity to the Man6*P*-receptor at the cell surface leading to an efficient uptake and transport to the lysosomes.

The successful use of scFv M6P-1 coupled to beads in immunoprecipitation experiments indicates that they may be of use in a purification procedure of Man6*P*-containing glycoproteins. Conveniently, a very mild elution of bound proteins would be achievable by the addition of Man6*P* or Fru1*P* without the risk of enzyme inactivation during the purification process. Such beads could also prove useful in studies aiming at the characterization of the Man6*P*-proteome of cells and organs as described for a soluble form of the Man6*P*-receptor purified from bovine serum (Sleat et al. 2007; Sleat et al. 2008; Sleat et al. 2009).

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