Monoclonal antibodies assisting refolding of firefly luciferase

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Abstract

The reactivation efficiency in the refolding of denatured luciferase in the presence and the absence of monoclonal antibodies (mAbs) has been studied. Luciferase could be partially reactivated when the protein was denatured in high concentrations of guanidium chloride (GdmCl; >4.5 M) and the refolding was carried out in very low protein concentrations. The refolding yield was, however, significantly lower when it was performed on luciferase that had been denatured with lower concentrations of GdmCl. The efficiency of refolding decreases when the formation of aggregates increases. Three of the five luciferase mAbs tested (4G3, N2E3, S2G10) dramatically increased the yield of reactivation and simultaneously eliminated the formation of aggregates. It is proposed that these mAbs assisted the refolding of luciferase by binding to the exposed hydrophobic surface of the refolding intermediate, thus preventing it from aggregating. The epitopes interacting with these refolding-assisting mAbs are all located in the A-subdomain of the N-terminal region of luciferase. These results have also shed light on the structural features of the intermediate and its interface involved in protein aggregate formation, contributing to the understanding of the protein folding mechanism.

Keywords: luciferase; protein unfolding and refolding; protein aggregation; monoclonal antibodies; epitopes; enzyme activity

The refolding of multidomain proteins is a more complicated process than that of single-domain proteins. During refolding, an improperly formed hydrophobic interface could increase the probability of aggregation. The off-pathway aggregation of proteins in the folding process is a ubiquitous, yet poorly understood process (Jaenicke 1995; Hor-

wich and Weissman 1997). The formation of abnormal aggregates and protein precipitates has been considered to be an obstacle in protein expression and might underlie the pathogenesis, in vivo, of some diseases of the neural system (Prusiner 1996). There is increased interest in exploring the mechanisms of protein aggregation. Protein aggregates were once considered to result from nonspecific interactions of unfolded or partially folded peptide chains. Increasing evidence from recent experimental results, however, suggests that aggregates might occur, in most cases, through the specific interaction of partially folded intermediate states with large exposed hydrophobic surfaces (Betts et al. 1997; Booth et al. 1997; Kelly et al. 1997; Fink 1998; Radford 2000). A number of studies have illustrated that aggregate formation is closely related to amino acid sequence (Wetzel 1994; Speed et al. 1996) and have suggested that the forces for aggregation are somewhat similar to those involved in

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonic acid; BSA, bovine serum albumin; CD, circular dichroism; Cm, midpoint; GdmCl, guanidinium chloride; mAb, monoclonal antibody.

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the folding and stability of globular proteins. In general, as most aggregates are insoluble and heterogeneous, spectroscopic methods such as fluorescence or CD could not be used. The use of mAbs is a powerful approach that can be used to study conditions preventing the formation of aggregates and thus, indirectly, help deduce its mechanism. For example, mAbs of reduced S-protein were reported to improve the yield of native protein during protein refolding, which suggests that mAbs may directly interact with specific epitopes during refolding (Carlson and Yarmush 1992).

mAbs have been considered a unique tool in the study of protein folding and aggregation. In the 1970s, Anfinsen initially proposed that antibodies could be a powerful tool to study protein folding (Anfinsen 1973). Subsequently, Furie et al. (1975) and Hurrell et al. (1977) successfully probed the local conformations of nuclease and myoglobin by using polyclonal antibodies. mAbs could bind to specific epitopes of a protein, thereby specifically providing information concerning a local conformational change involved in protein folding and aggregation. Goldberg's group prepared several conformation-specific and sequence-specific mAbs to study the refolding process and ligand-induced conformational changes of *Escherichia coli* tryptophan synthetase (Friguet et al. 1984; Fedorov and Baldwin 1997).

Hattori and colleagues (1993) reported that anti- β -lactoglobulin mAbs could be used to monitor local conformational changes and to differentiate between conformations in the denatured and native forms of this protein. In addition, mAbs have been used to identify intermediates in the aggregation pathway of P22 tailspike polypeptide chains (Friguet et al. 1994; Speed et al. 1997).

Firefly luciferase (Luc) from *Photinus pyralis* catalyzes the oxidation of luciferin with molecular oxygen in the presence of ATP and Mg^{2+} . This reaction results in luminescence emitted at 560 nm (de Wet et al. 1987). Luciferase is a monomeric protein with a molecular weight of 62 kDa. The crystal structure of luciferase was solved in 1996 and comprises two globular domains, the N- and C-terminal domains (Conti et al. 1996). The N-terminal domain can be further divided into three subdomains, A (residues 77–222 and a loop of 399–405), B (residues 22–70 and 236–351), and C (residues 4–10, 363–393, and 418–434). From previous work (Xu et al. 1999), we obtained five mAbs against luciferase. Competitive binding experiments have shown that two mAbs can bind to the heat-denatured antigen and its proteolytic fragments but not to native luciferase, thus suggesting that their epitopes might be located in the internal segments of the protein. The other three mAbs can bind to both the native and the denatured enzymes. The five mAbs are all sequence specific. Using these antibodies and various spectroscopic methods, we studied the unfolding/refolding process of luciferase and found that three of the five mAbs dramatically increased the refolding yield and simultaneously eliminated the formation of aggregates.

These observations support the proposition that improper interactions between partially structured intermediates of the refolding of luciferase led to protein aggregation. Moreover, analysis of their epitopes provided clues regarding the structural features of the intermediate and its interface involved in protein aggregation.

Results and Discussion

Equilibrium unfolding

The GdmCl-induced unfolding process of luciferase was followed by enzyme activity, intrinsic fluorescence, CD spectra, and ANS-binding fluorescence (Fig. 1). The curve for activity loss against the concentration of GdmCl was approximately sigmoid. Complete inactivation of the enzyme activity occurred at a concentration >0.5 M GdmCl. The midpoint of concentration for GdmCl denaturation ($C_{1/2}$) occurred at ~ 0.35 M. The decrease in intrinsic fluorescence was multiphasic. The first stage occurred over the range of 0.15–0.5 M GdmCl, where the fluorescence intensity dropped drastically. The fluorescence change curve at this stage (below 0.5 M GdmCl) mirrored the activity loss, indicating that the activity loss was accompanied by conformation changes with exposure of the aromatic chromophores to the solvent. The second stage consisted of a plateau occurring between 0.5 and 1.4 M GdmCl, followed by a third stage (1.4–2.5 M GdmCl) where the fluorescence decreased to the baseline. ANS fluorescence was somewhat different. From 0 to 0.5 M GdmCl, the ANS fluorescence increased drastically. The ANS fluorescence remained at this high intensity level between 0.5 and 1.2 M GdmCl and subsequently dropped when the concentration of GdmCl reached the range of 1.2–2.5 M. Far-UV CD ellipticity at 222 nm was also biphasic. The first decline in CD occurred at 0.7–1.2 M GdmCl, and the second decline was evident at

Figure 1. The equilibrium unfolding transition of luciferase detected by activity (triangle), intrinsic fluorescence (inverted triangle), ANS-binding fluorescence (square), and CD ellipticity at 222 nm (circle).

3.5–4.5 M GdmCl. A noticeable lag could be observed in the CD ellipticity curve in comparison to that of intrinsic fluorescence and ANS fluorescence.

Reactivation of luciferase

The recoverable activities of luciferase plotted against the GdmCl concentration in which the enzyme had been unfolded, and from which the refolding dilution was made, showed a U-like profile (Fig. 2A). Very low reactivation efficiencies were obtained when luciferase had been denatured at moderate concentrations of GdmCl (0.7 M–4.5 M). However, when the enzyme had been denatured in higher concentrations of GdmCl (>4.5 M), the activity was partially restored. To further understand whether the low refolding efficiencies were caused by protein aggregation during refolding, we determined the concentration of protein remaining in the supernatant of the refolding solution. As shown in Figure 2B, the profile of the amount of soluble protein against the concentration of GdmCl is also U-like. This indicates that most of the enzyme protein was dena-

Figure 2. (A) The refolding yield of luciferase. Reactivation of the enzyme was performed after incubation of the protein for 3 h in various concentrations of GdmCl as indicated on the X-axis. Refolding was induced by dilution of the denatured enzyme to 0.05 M GdmCl and a protein concentration 6 $\mu\text{g/mL}$ using buffer I. (B) Concentration of soluble protein in the reactivation solution of luciferase diluted from different concentrations of GdmCl after centrifugation. The total protein concentration before reactivation is taken as 100%.

Figure 3. Kinetics of the luciferase reactivation process. (Inset) The semi-log plot of luciferase reactivation is linear.

tured at moderate concentrations of GdmCl, and failed to refold with the formation of aggregates. Luciferase, unfolded at moderate concentrations of GdmCl, may exist as an intermediate state with a greater tendency to form aggregates. The reactivation kinetics from a completely unfolded state followed first-order kinetics (Fig. 3) with a first-order reaction constant of $2.51 \times 10^{-4} \text{ sec}^{-1}$. The reactivation of luciferase was unusually slow for a mono-sub-unit protein.

MAb-assisted reactivation of luciferase

The reactivation of luciferase in the presence of each of the five mAbs (molar ratio 1:1) is presented in Figure 4. The reactivation efficiency of completely unfolded luciferase, regardless of the presence or absence of BSA, was dependent on the protein concentration used. It was found to be very low at higher protein concentrations, supporting the existence of an off-pathway aggregation. When refolding was performed in the presence of 4G3, N2E3, or S2G10, the reactivation efficiency was clearly improved (4G3 and N2E3 could assist the full recovery of luciferase activity, whereas S2G10 could only improve the recovery of activity to 80%, Fig. 4). The mAbs-assisted refolding was independent of the concentration of luciferase. Antibodies 2H5 and 3E7 had no apparent effect on the refolding process (Fig. 4). The molar ratio of mAbs to luciferase had an influence on the activity recovery, as shown in Figure 5. The maximum reactivation occurred at a molar ratio >0.7 (ratio of mAbs to luciferase).

By incubating each antibody with active luciferase, under the same conditions as used in the reactivation process, we determined that none of the five mAbs affected luciferase activity. It excluded the possibility that certain mAbs can improve reactivation simply as a result of the improvement of luciferase solubility by binding to such a large molecule.

Figure 4. Effects of five mAbs on the refolding yield of luciferase. Luciferase was incubated in 6 M GdmCl for 3 h before refolding. Refolding was started by dilution of the denatured enzyme in the absence or presence of BSA or of indicated mAb. The GdmCl concentration during refolding was 0.05 M. 4G3 (triangle, *D*), N2E3 (circle, *C*), and S2G10 (square, *B*) improve the reactivation efficiency of the denatured luciferase. 2H5 (inverted triangle, *E*) and 3E7 (diamond, *F*) had no effect on the refolding yield of luciferase in the presence of BSA (right triangle, *H*) and in the absence of BSA (left triangle, *G*).

Epitope analysis

S-carboxymethyl-luciferase digested by TPCK-trypsin was separated by HPLC using a C18 reverse column. The peptides containing epitopes for four mAbs (4G3, N2E3, S2G10, 2H5) were identified by dot blot. The peptide containing the epitope for 3E7 was derived by Glu-C digestion.

Figure 5. The reactivation efficiency correlates to the molar ratio of mAb and refolded luciferase. 4G3 (inverted triangle) and N2E3 (triangle) and S2G10 (square), and control (circle; BSA in equivalent molar ratio). The refolding luciferase concentration is 6.5 $\mu\text{g/mL}$.

The sequences of the five mAbs were determined by mass spectroscopy combined with amino acid sequencing analysis as listed in Table 1.

Determination of the structural features of the epitopes of the three refolding assisting mAbs in the 3D structure of luciferase

The interfaces between the epitopes of the three refolding-assisting mAbs and the corresponding regions of luciferase were assigned according to the atomic distance in the crystal structure. The cutoff value was set at 3 Å, as this is the average distance of a hydrogen bond. The residues in contact with the S2G10 epitope were all located in the A-subdomain (Arg 188, Asp 189, Thr 191, Ala 193, Leu 194, Met 196, Pro 395, Ser 399, and Val 402). The residues interacting with the N2E3 epitope were also located in the A-subdomain (Val 102, Ser 117, Asp 187, Val 208, Leu 210, and His 212), with the exception of Glu 344. The 4G3 epitope could interact with residues in both the A-subdomain (Lys 148, Ile 151, Lys 154, and Phe 181) and partial B-subdomain (Lys 31 and Glu 58).

On the basis of the sequence of the epitopes, the structural features of the peptide segments in luciferase are further illustrated in Figure 6. The 4G3 epitope, consisting of an α -

Table 1. *The properties of the five mAbs*

	S2G10	2H5	4G3	N2E3	3E7
Effect on luciferase activity	none	none	none	none	none
Antigen binding	native and denatured	native and denatured	native and denatured	denatured only	denatured only
Improvement of refolding efficiency	yes	no	yes	yes	no
Epitope location peptide	207–213	514–524	156–180 ^a	190–205 ^a	345–370
Molecular weight of peptide (MS)	749.0	1145.0	4166.0	2702.0	2689.0
Molecular weight of peptide (calculated)	749.0	1145.0	4166.0	2702.6	2689.6
Peptide sequence	GVALPS	GGVVFV	F(T,A)DYQG F	DFVTH	M(K)Q(T)HA ILTP
Sequence reported	GVALPH	GGVVFV	TDYQGF	DFVPE ^b	TTSAILITP

^a The peptides 156–190 and 180–205 in 4G3 and N2E3 were analyzed by both MS and amino acid sequencing. Two mAbs did not show any cross-reaction with the epitope containing peptides in the dot blot; it may be deduced, then, that the overlapping segment is unlikely to be involved in the mAb's epitopes.

^b The underlined letters represent the residues that did not agree with the sequence reported.

helix and a long loop, was on the surface of the A-subdomain, at the edge of the interface between the A- and B-subdomains (Fig. 6, upper left). The epitopes of S2G10 and N2E3 were continuous in amino acid sequence and constituted two adjacent antiparallel β -strains of the A-subdomain (Fig. 6, upright). On the basis of the X-ray structure data, the two β -strains formed a part of a hydrophobic core in luciferase (Fig. 6, middle left). We calculated the relative solvent accessibility surface area of the residues in the N2E3 epitope in the native state and found that the main part of this peptide segment was internally embedded in the protein (Fig. 6, middle right), which was consistent with the competitive binding experiments. The epitopes for the remaining two mAbs (3E7 and 2H5), which had no detectable effects on the refolding, were located between the B- and C-subdomains and on the surface of the C-terminal domain, respectively. The 3E7 epitope participated in the interaction between these two subdomains and was not accessible in the native conformation (Fig. 6, lower panel), as previously indicated by competitive binding experiments.

Whereas some small single-domain proteins have been shown to fold without detectable intermediates, many larger (especially multidomain and oligomeric) proteins are known to form intermediates during their folding processes, often leading to aggregation as a side reaction. We have shown that the unfolding of firefly luciferase under equilibrium conditions has at least three transitions. This fact may reflect the complexity of the luciferase structure, as it is composed of multiple structural levels (domains and subdomains). The reactivation rate of luciferase is unusually slow in comparison to a monomeric small protein whose folding rate is in the order of milliseconds. As the completion of fluorescent changes was achieved in seconds (data not shown), it is reasonable to suggest that the unfolded luciferase rapidly folds into a native-like intermediate state, followed by a slow conformational adjustment process to recover the activity, which may be the rate-limiting step. As the active site of luciferase is located on the surface between

the N- and C-terminal domains, it is possible that the domain reorganization is involved in the rate-limiting step of reactivation.

The “refolding trough” strongly correlates with the formation of aggregates, suggesting that the partially folded intermediate has a greater tendency to aggregation. It is proposed that the large hydrophobic surfaces are exposed in these intermediates during the unfolding process and the improper intermolecular interaction of these surfaces leads to aggregation. When refolding is performed on completely unfolded luciferase (denatured in GdmCl concentrations higher than 4.5 M), the activity could be partially restored and the reactivation yield is dependent on the concentration of refolding protein. This result suggests that the kinetic competition between the productive refolding and improper aggregation pathways determines the yield of the active enzyme.

There has been some controversy about whether the formation of aggregates is specific (Trivedi et al. 1997). The fact that three mAbs are able to increase the refolding efficiency and completely eliminate the formation of aggregates, whereas two mAbs were ineffective, supports the proposal that the special surface of the intermediate may be responsible for aggregate formation, at least in this case. The curve of activity recovery against the molar ratio of these three reactivation-assisting mAbs to luciferase appears as a typical saturation curve, which indicates that the interaction between mAbs and luciferase is critical to the refolding-assisting effect.

The three refolding-assisting mAbs are all sequence specific, with N2E3 specifically binding to the heat-denatured antigen. It is unlikely that these mAbs assist in the reactivation of luciferase by stabilizing the native state. Moreover, these refolding assisting mAbs do not accelerate the refolding rate but do effectively improve the refolding yield. Therefore, the assistance to refolding is unlikely to be a catalytic reaction. The most probable explanation is that the mAbs bind to the specific interface of the partially refolded

Figure 6. (Top left) Location of the 4G3 epitope in the 3D structure of luciferase (only the A- and part of the B-subdomains are shown). The protein secondary structure is shown as a schematic model, and the peptide, as an atomic model. The residues in the B-subdomain that make contact with the peptides are shown in green in the atomic model. (Middle left) Location of the epitopes for S2G10 (blue) and N2E3 (green) in the luciferase 3D structure (only the A-subdomain is shown). The A-subdomain is shown schematically. (Top right) The epitopes for S2G10 (orange) and N2E3 (green) are located in the hydrophobic core of the A-subdomain. The hydrophobicity of the secondary structure is represented by different colors. The colors white, blue, pink, and red represent a decreasing hydrophobicity. (Middle right) Relative solvent-accessible surface area of the residues in N2E3 epitope in native state. The calculation was completed using GETAREA 1.1, a Web service provided by the Sealy Center for Structural Biology at the University of Texas Medical Branch. Relative solvent-accessible surface area accessibility was defined as the ratio of side-chain surface area to “random coil” value per residue. The random coil value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations. Ratios larger than a value of 20%, as a default value, were considered as exposed residues. (Bottom left) Location of the 3E7 epitope in the 3D structure of luciferase (only the B- and part of the C-subdomains are shown). The protein structure is shown as a schematic model and the peptide as an atomic model.

intermediate state of luciferase. The formation of the mAb complex with the refolding intermediate produces a very low concentration of free intermediate, which spontaneously precedes the refolding process. Because of the large

size of the antibody molecule, we cannot rule out the possibility that steric hindrance caused by the mAbs may also prevent the formation of aggregates. We propose that specific interaction of the mAbs plays an important role. This

is particularly illustrated in the case of N2E3, because the N2E3 epitope is adjacent to that of S2G10 in the primary sequence, but their refolding-assisting efficiencies are clearly different (under saturating concentrations, N2E3 can assist luciferase in recovering full activity, whereas S2G10 only improves the activity recovery to a level of 80%). This indicates that the epitope for N2E3 is more critically involved in aggregate formation, whereas S2G10 may act by binding near the specific interface of the intermediate. We tentatively suggest that the mechanism for mAb assisting-refolding is similar to that of Hsp 70, which binds to the hydrophobic patches of unfolded or partially folded peptides (Feldman and Frydman 2000).

From the results of the epitope mapping, we have obtained information pertaining to features of the refolding intermediate as well as important information concerning the aggregate-forming segment of luciferase. The epitopes of the three refolding-assisting mAbs are all located in the A-subdomain of luciferase, which may imply a critical role for this structural domain in aggregation. The epitopes of S2G10 and N2E3 constituted part of the hydrophobic core of luciferase according to the X-ray diffraction data. It is reasonable to deduce that hydrophobic interaction of these peptides, which are exposed in the intermediates during the refolding of luciferase, would cause the formation of aggregates. The present approach could be used for studies of disease-related aberrant protein folding, which might provide valuable information for rational drug design.

The mAbs, which had no effect on the reactivation process, could also be informative regarding the mechanism of refolding. For example, the 3E7 epitope is located in the interface between the B- and C-subdomains and is highly hydrophobic. As 3E7 does not assist in the refolding, it is possible that most of the relevant portion of the B- and C-subdomains could be buried prior to the formation of the partially folded intermediate.

On the basis of this discussion, we propose a refolding mechanism for luciferase: The interface of the B- and C-subdomains is engulfed first before the A-domain is properly folded. The adjustment in the conformation of the A-subdomain and hiding of the hydrophobic segment in the interface between the A- and B-subdomains might be the rate-determining step in luciferase refolding. Further details concerning the folding and aggregation process of luciferase might be illustrated by acquiring additional mAbs with different specificities.

Materials and methods

Materials

Luciferase and mAbs were purified as described previously (Fedorov and Baldwin 1997). Ultra pure GdmCl was purchased from USB. TPCK-trypsin and Glu-C were Sigma products. BCIP and

NBT were from Pharmacia. ANS was from BDH. Other chemicals were analytical grade.

Luciferase assay

The enzyme was dissolved in 50 μ L buffer I (100 mM Tris-HCl, 200 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT at pH 7.8) containing 1 mM luciferin. The total volume was adjusted to 100 μ L with buffer I. The cuvette was placed in an FG-300 luminometer at room temperature. The reaction was initiated by mixing 400 μ L of buffer II (containing 2.5 mM ATP, 5 mM MgSO₄ in buffer I). The luminescence was recorded by photon counting. The enzyme activity was calculated from photon counts. Enzyme assay during unfolding and refolding was performed in a buffer containing the same concentration of denaturant. Protein concentration was determined spectrophotometrically at 280 nm by using a molar extinction coefficient of $3.72 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

GdmCl-induced unfolding of luciferase

Luciferase was incubated in buffer I containing defined concentrations of GdmCl for about 3 h at 20°C to reach equilibrium. In parallel control experiments, luciferase displayed no detectable changes in activity or spectroscopic properties over the same incubation intervals. The protein concentration was $1.6 \times 10^{-6} \text{ M}$ for the CD measurements, and $3.2 \times 10^{-7} \text{ M}$ for the fluorescence measurements. The concentration of GdmCl was calibrated by its refractive index as described by Nozaki (1972).

Intrinsic fluorescence

The fluorescence was determined by a fluorophotometer (Hitachi F-4500) at 20°C. The excitation wavelength was set at 280 nm, and the emission fluorescence was recorded at 340 nm. The fluorescence intensity was corrected with an appropriate blank and normalized to an arbitrary unit.

ANS-binding fluorescence

Luciferase was incubated in a series of concentrations of GdmCl in the presence of 50-fold molar excess of ANS for 1 h at 20°C in the dark, and then the fluorescence was measured at 480 nm at an excitation wavelength of 350 nm. Appropriate blank subtractions of ANS and GdmCl were made for the fluorescence.

Circular dichroism

Far-UV CD measurements were performed on a JASCO-715 spectropolarimeter with a computer data processor. Spectra were recorded over 190–250 nm in a 1-mm pathlength cuvette at a scan speed of 10 nm/min with a time constant of 0.25 sec. Data were further processed for noise reduction, baseline subtraction, and signal averaging when necessary. The secondary structure contents were estimated by ellipticity at 222 nm.

Reactivation of luciferase

Luciferase was incubated with various concentrations of GdmCl (0.1 M–5.5 M) for 3 h at 20°C. Refolding was initiated by dilution of the denatured enzyme to 0.05 M GdmCl and a final protein concentration of $9.6 \times 10^{-8} \text{ M}$ with buffer I. Aggregates in the

refolding mixture were removed by centrifugation. The protein concentration of the supernatant was determined by the Bradford method. Reactivation kinetics was followed by monitoring the activity of enzyme aliquots at different time intervals.

MAb-assisted reactivation of luciferase

Luciferase unfolded by 6 M GdmCl was diluted 120-fold with buffer I containing mAb to a final concentration of 0.05 M GdmCl. In the control experiment, luciferase was incubated and diluted under the same conditions except that the same amount of BSA instead of mAb was added to the dilution buffer.

Epitope mapping

Peptide fragments were obtained by hydrolysis of S-carboxyl-methyl luciferase with TPCK-trypsin or Glu-C (1:100 ratio of luciferase to trypsin and 1:50 ratio of luciferase to Glu-C) for 24 h (trypsin) or 48 h (Glu-C). The protease was inactivated by boiling at 100°C for 10 min. Isolation of peptides was achieved by HPLC using a C18 reverse column. Peptides containing epitopes were characterized by dot blot on a PVDF membrane. The peptide fragment that yielded a positive reaction to the mAb was then subjected to mass spectroscopy and protein sequencing analysis.

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