Kinetics of Nonproteolytic Incorporation of a Protein Ligand into Thermally Activated α2-Macroglobulin

EVIDENCE FOR A NOVEL NASCENT STATE*

We have previously shown that antigens complexed to the receptor-recognized form of α2-macroglobulin (α2M*) demonstrate enhanced immune responsiveness mediated by the low density lipoprotein receptor-related protein LRP/CD91. Recently, we developed a proteinase-independent method to covalently bind antigens to α2M*. Given the potential applications of this methodology, we analyzed the kinetics, thermodynamics, and pH dependence of this reaction. The incorporation of lysozyme into α2M* was a mixed bimolecular second-order reaction with a specific rate constant of 91.0 ± 6.9 M⁻¹ s⁻¹, 50.0 °C, pH 7.4. The activation energy, activation entropy, and Gibbs’ free energy at 50.0 °C were 156 kJ mol⁻¹, 266 J mol⁻¹ K⁻¹, and 70 kJ mol⁻¹, respectively. The rate of incorporation increased as a function of pH from 5.0 to 7.0 and was unchanged thereafter. Furthermore, the reaction between α2M* and lysozyme was irreversible. The data are consistent with a two-step mechanism. In the first step, α2M* reforms its thiol ester bond, entering a reactive state that mimics the proteolytically induced “nascent state.” In the rate-limiting second step, the reformed bond quickly undergoes nucleophilic attack by lysozyme. The kinetic equations derived in this study are the basis for optimizing the formation of stable α2M*-antigen complexes.

Human α2-macroglobulin (α2M) is a 720-kDa tetrameric glycoprotein that can inhibit proteinases of all classes and specificities through a unique mechanism involving both steric entrapment and covalent binding (1). α2M is composed of four identical subunits that form a cage-like structure (2). Each subunit contains a “bait region,” a stretch of amino acids that is highly susceptible to proteinase cleavage (3), and a functionally important internal β-cysteinyl-γ-glutamyl thiol ester (4–6). In native α2M, the thiol ester bond is composed of the side chains of Cys949 and Glx952 and appears to sit in a protective hydrophobic pocket that restricts access of most nucleophiles (4–6). Limited proteolysis of the bait region activates the thiol ester, resulting in a transient “nascent state” in which the thiol ester bond is extremely susceptible to nucleophilic substitution. Subsequently, a major conformational change occurs in α2M, yielding the more compact receptor-recognized form (α2M*) (7). The covalent attachment of the proteinase to α2M occurs between Glx952 of the nascent state thiol ester and an available lysine or NH₂ terminus of the proteinase. Other proteinases, if present during proteolysis, can react with the thiol ester and may compete with the proteinase for covalent binding (8, 9).

Although traditionally viewed as a proteinase inhibitor, α2M is also a major serum carrier of cytokines and growth factors and has been implicated in the regulation of the immune system. Our laboratory has demonstrated that covalently linking an antigen to α2M* enhances uptake, processing, and presentation of that antigen by peritoneal macrophages (10). Macrophages pulsed with lysozyme complexed to α2M* achieved effective presentation to T cells with 200- to 250-fold less protein than cells pulsed with free lysozyme. This enhancement was mediated by the α2M* receptor, the low density lipoprotein receptor-related protein LRP/CD91. These studies were extended in vivo by demonstrating that, compared with free lysozyme, lysozyme complexed to α2M* resulted in 10- to 500-fold higher IgG titers (11). These levels were similar to those elicited by emulsification in Complete Freund’s Adjuvant, generally considered the most potent adjuvant currently available.

The α2M*-antigen complexes were generated by co-incubating an antigen with α2M* in the presence of a proteinase (12). This method for complex formation results in complexes that contain varying amounts of proteinase that compete with the antigen for binding to the thiol ester. Although some antigen is covalently incorporated inside α2M*, the trapped proteinase remains functional and is sometimes able to destroy the antigen. Additionally, if these complexes are used for induction of an immune response, they may lead to the generation of antibodies against the proteinase (11).

We have recently developed a method for incorporating non-proteolytic proteins into α2M* without the use of a proteinase (13). We observed that NH₃ or CH₃NH₂-activated α2M, when heated, can covalently bind to co-incubated proteins. The absence of the proteinase within the α2M* cage allows more and larger antigens to incorporate into α2M* (13). More recent studies from our laboratory using complexes generated in this manner have demonstrated a remarkable increase in both the humoral and T-cell-mediated immunogenicity against the hepatitis B surface antigen and the HIV peptide C4-V3 (14).

As a result of the recent interest in LRP/CD91-mediated antigen presentation, the adjuvant-like properties of α2M* are being increasingly studied.
have been receiving increasing attention. The optimization of α₂M-antigen complex formation hinges upon the chemistry of this reaction. Recent attempts to create these complexes using nonproteolytic activation, however, have resulted in poor antigen incorporation (15). Detailed kinetic analysis presented here allows for the optimization of antigen incorporation into α₂M. By appropriate use of this chemistry, significant amounts of antigen can be incorporated into α₂M.

EXPERIMENTAL PROCEDURES

Preparation of α₂M and 125I-HEL—Human α₂M was purified from frozen human plasma (American Red Cross, Charlotte, NC) according to a previously published protocol (16). The concentration of α₂M was determined using A₂⁸₀ (1%/1 cm) = 8.93 and a molecular mass of 720 kDa (17). Two commercial α₂M preparations were purchased from Sigma-Aldrich. The thiol ester-cleaved α₂M derivative was prepared by incubating native α₂M with 0.2 m NH₄CO₃, pH 8.0, for 60 min at room temperature. Excess NH₄ was removed by size-exclusion chromatography on Sephadex G-25 (Amersham Pharmacia Biotech) equilibrated in 0.1 m NaPi and 0.15 m NaCl (PBS), pH 7.4. The efficiency of the conversion to α₂M* was determined by following its electrophoretic mobility on non-denaturing, nonreducing polyacrylamide 4–15% Tris-HCl gels (Bio-Rad, Hercules, CA) (7). HEL (Sigma-Aldrich) was iodinated with Iodogen-coated tubes (Pierce) and Na¹²⁵I (PerkinElmer Life Sciences). Typically, 2.5 mg of HEL was incubated with 1.0 mCi of ¹²⁵I in 500 µl of PBS, pH 7.4, for 30 min at room temperature. Residual ¹²⁵I was removed on Sephadex G-25 equilibrated in PBS, pH 7.4. The concentration of lysozyme was determined using A₂⁸₀ (1%/1 cm) = 26.5, assuming a molecular mass of 14 kDa (18). Both α₂M* and 125I-HEL were sterile-filtered through Millex-GV 0.22 µm filter units (Millipore) and stored at 4 °C in PBS, pH 7.4. Proteins were used within 2 weeks of conversion (α₂M*) or iodination (125I-HEL).

Measurement of 125I-HEL Incorporation into α₂M*—The kinetics of ligand incorporation were measured using 125I-HEL as a model ligand, as it has been in previous studies involving the incorporation of ligands into α₂M with and without the use of proteinase (10, 11, 13). All measurements of kinetics were performed during the first 10 min of the reaction, whereas experiments requiring reaction completion were performed for 180 min. Varying amounts of α₂M* (0.025–0.2 µM) were incubated with 125I-HEL (2.5–20 µM) at 50.0 °C for the appropriate time (2.5–180 min) in PBS, pH 7.4. Incubations were performed in a water bath, the temperature of which was monitored with a digital thermometer (Control Company, Friendswood, TX) with an accuracy of ±0.1 °C. After incubation, reaction mixtures were subjected to non-denaturing, nonreducing, pore-limit polyacrylamide gel electrophoresis and nonreducing SDS-polyacrylamide gel electrophoresis to determine the amount of total and covalent 125I-HEL that was bound to α₂M*, respectively. Electrophoresis was performed on 4–15% Tris-HCl gels (Bio-Rad) that were stained, dried, and analyzed on a Molecular Dynamics STORM 860® (Sunnyvale, CA). 125I-HEL incorporation into α₂M was analyzed by measuring the amount of 125I-HEL associated with the band corresponding to the molecular weight of α₂M* using ImageQuant 5.1® (Molecular Dynamics). Linear and nonlinear regressions were performed using Jandel-Scientific SigmaPlot® (Chicago, IL). All noncovalent 125I-HEL binding (determined by subtracting the amount of 125I-HEL covalently bound from the total 125I-HEL bound) was determined to be nonspecific due to its linear, nonsaturating nature.

To determine whether the approach employed to terminate the reaction affected the binding kinetics, two different methods were compared. Reactions were stopped either by cooling on ice or by adding SDS at a temperature of 100 °C and immediately incubating the mixture at 100 °C for 10 min. No difference in the rate of 125I-HEL incorporation into α₂M was observed, and all subsequent reactions were stopped on ice.

Temperature and pH Studies of 125I-HEL Incorporation into α₂M*—The temperature dependence of the rate of incorporation of 125I-HEL into α₂M was determined between 47.5 °C and 55.0 °C at pH 7.4. The reactants were incubated at 125I-HEL:α₂M ratios of 20.0:2 µM, 10.0:2 µM, 20.0:1 µM, and 10.0:1 µM at four temperatures (47.5 °C, 50.0 °C, 52.5 °C, and 55.0 °C). Samples were collected at 2, 2.5, 5.0, 7.5, and 10.0 min and then analyzed. Specific rate constants were computed at each temperature using the experimentally derived rate equation. The activation energy (Eₐ) of the reaction was analyzed with the Arrhenius equation:

$$k = \frac{Ae^{-\frac{E_a}{RT}}}{T}$$

(Eq. 1)

where kₚ is the Boltzmann constant (1.38 × 10⁻²³ J/K), h is Planck’s constant (6.626 × 10⁻³⁴ J/s), and T is the average temperature. The Gibbs’ free energy (ΔG°) at 50.0 °C was analyzed with the equation below (ΔH*, activation energy; ΔS*, activation entropy).

$$ΔG° = ΔH° - TΔS°$$

(Eq. 3)

The effect of pH on the reaction rate of 125I-HEL incorporation into α₂M* was studied between pH 5 and pH 9. α₂M* and 125I-HEL were brought to pH 5.0, 6.0, 7.0, 8.0, or 9.0 in 0.1 m NaPi and 0.15 m NaCl through buffer exchange using Amicon® Micron® YM-10 and YM-3 filters (Millipore), respectively. 125I-HEL binding was measured at 2.5, 5.0, and 10.0 min. Specific rate constants were calculated for each pH studied. The amount of 125I-HEL incorporated at equilibrium was measured at 180 min at a 125I-HEL:α₂M* ratio of 100:1.

RESULTS

Time and Concentration Dependence of 125I-HEL Incorporation into α₂M*—Studies described below indicated that the incorporation of 125I-HEL into α₂M* at 180 min saturated at a 125I-HEL:α₂M* ratio of ~100:1; therefore, binding was performed under these conditions to observe the reaction time course. The incorporation data fit a monophasic exponential curve that was linear for 15–20 min and approached equilibrium at 180 min (data not shown). The relationship between 125I-HEL incorporation into α₂M* and the ratio of the concentrations of the reactants was then measured after 180 min of incubation at 50.0 °C, with 125I-HEL:α₂M* molar ratios ranging from 1.5–200. The plot of initial 125I-HEL concentration versus the amount of 125I-HEL bound to α₂M* demonstrated maximal 125I-HEL incorporation at a 100-fold molar excess of 125I-HEL (20 µM) (Fig. 1).

Rate Equation for 125I-HEL Incorporation into α₂M*—The order of the reaction with respect to 125I-HEL and α₂M was calculated using the method of initial rates. To determine the order with respect to α₂M*, 125I-HEL (20 µM) was combined with varying concentrations of α₂M* (0.25–2 µM) at 50.0 °C, and samples were collected and analyzed between 2.5 and 10.0 min (Fig. 2A). The plots of 125I-HEL concentration versus the amount of 125I-HEL bound confirmed that the reaction was in the linear phase. The logarithm of the initial rate was directly proportional to the logarithm of the...
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\(\alpha_2\)M\(^\ast\) concentration (Fig. 2B), and the slope of the linear regression was 1.02 ± 0.09, suggesting that the reaction was first order with respect to \(\alpha_2\)M\(^\ast\). This procedure was repeated to determine the order of 125I-HEL and yielded similar results (Fig. 2, C and D). The concentration of \(\alpha_2\)M\(^\ast\) was held constant at 0.2 \(\mu\)M, whereas 125I-HEL concentration varied from 2.5–20 \(\mu\)M. The relationship between the logarithm of the reaction rate and 125I-HEL concentration was also linear with a slope of 1.08 ± 0.13, indicating that the reaction was first order with respect to 125I-HEL. Using the rate equation and the orders of 125I-HEL and \(\alpha_2\)M\(^\ast\), the specific rate constant \(k\) at 50°C was calculated as 91.0 ± 6.9 \(\text{M}^{-1}\text{s}^{-1}\) and was independent of the reactants’ concentrations. Therefore, the experimentally derived rate equation for covalent incorporation of 125I-HEL into \(\alpha_2\)M\(^\ast\) can be expressed by the relationship:

\[
\frac{d[125I-\text{HEL}:\alpha_2\text{M}\^{\ast}]}{dt} = 91.0 \frac{1}{\text{M}\text{s}} [125I-\text{HEL}][\alpha_2\text{M}\^{\ast}]
\]

(Eq. 4)
in which \(\alpha_2\)M\(^\ast\) represents an individual subunit of the \(\alpha_2\)M tetramer.

Temperature and pH Dependence of 125I-HEL Incorporation into \(\alpha_2\)M\(^\ast\)—The temperature dependence of the rate of 125I-HEL incorporation into \(\alpha_2\)M\(^\ast\) was determined from 47.5°C to 55.0°C at pH 7.4. Samples were collected and analyzed at 2.5, 5.0, 7.5, and 10.0 min. Specific rate constants were computed at each temperature using the experimentally derived rate equation. The Arrhenius plot of the data demonstrates a linear relationship between \(\ln k\) and the inverse of temperature within the limited temperature range studied (Fig. 3 and Table I). From the plot, the following thermodynamic parameters were calculated: \(\Delta H^\circ = 156 \text{kJ mol}^{-1}\), \(\Delta S^\circ = 266 \text{J mol}^{-1} \text{K}^{-1}\), and \(\Delta G^\circ\) at 50.0°C = 70 \text{kJ mol}^{-1}.

Analysis of the effect of pH on the reaction rate of 125I-HEL incorporation into \(\alpha_2\)M\(^\ast\) was carried out over the pH range of 5.0–9.0. The plots of the amount of 125I-HEL incorporated versus time are linear at all pH values investigated during the time interval studied. Therefore, the reaction order increased as pH was raised from 5.0 to 7.0. No change was observed as pH increased from 7.0 to 9.0 (Table II). Measurements of the amount of 125I-HEL incorporated after 180 min demonstrated a similar amount of binding for all pH values (data not shown).

**Stability of 125I-HEL-\(\alpha_2\)M\(^\ast\) Complexes**—It has been observed that the reaction between the thiol ester and the nucleophiles ammonia and methylamine can be reversed at high temperatures (19). To determine whether nonproteolytic incorporation...
was also reversible, $^{125}$I-HEL-$\alpha_M^*$ complexes were generated by incubating a 100-fold excess of $^{125}$I-HEL with $\alpha_M^*$ for 180 min at 50.0 °C. Free $^{125}$I-HEL was removed by five washes through YM-100 filter units, and the purified complexes were heated to 50.0 °C. Samples were collected at various times between 5 and 180 min, and the amount of $^{125}$I-HEL complexed to $\alpha_M^*$ was compared with the total amount of $^{125}$I-HEL complexed at time 0 (Fig. 4). After 180 min of incubation at 50.0 °C, the amount of $^{125}$I-HEL bound to $\alpha_M^*$ was unchanged, indicating that the $\alpha_M^*$-$^{125}$I-HEL complexes were intact and that the incorporation was irreversible. This result is in marked contrast to the reversibility of the reaction of small nucleophiles incorporated into the thiol ester of $\alpha_M^*$ (19).

**Discussion**

Our laboratory has previously demonstrated that $\alpha_M^*$ significantly enhances antigen processing and presentation by carrying antigen into macrophages through a receptor-mediated process involving LRP/CD91 (10). Compared with free HEL, there was a 5-fold increase in complex uptake but a 100-1000-fold increase in T-cell response (10). These studies demonstrate that the enhanced responsiveness results from enhanced antigen presentation by antigen-presenting cells and is not simply dependent on an increase in $\alpha_M^*$-HEL complex uptake. A potent B-cell response was observed in vivo and in vitro with 10- to 500-fold higher IgG titers toward HEL when it was complexed to $\alpha_M^*$ (11). We have recently developed a novel method of covalent attachment of ligands to $\alpha_M^*$ without the addition of proteases. Studies have demonstrated that complexing antigens to $\alpha_M^*$ in this manner can increase the antigen-specific B- and T-cell responses. More recent studies by other investigators propose a broader role for LRP/CD91 in antigen presentation, suggesting that the receptor may also take up and process antigens coupled to various heat shock proteins (20). When the available literature is considered, it is likely that $\alpha_M^*$ and other molecules carrying antigens employ LRP/CD91 as an entry point for antigen delivery through multiple pathways.

In general, host response toward protein subunit antigens is poor. It is therefore likely that $\alpha_M^*$ will be employed to produce complexes with candidate subunit vaccine antigens to boost the responsiveness of human recipients toward these preparations. The $\alpha_M^*$ employed for such purposes will not be generated by proteases for a variety of reasons. First, to incorporate antigens into $\alpha_M^*$, the antigen must be present during the activation step. Nonproteolytic proteins can only form covalent adducts if they are present during the nascent state resultant from attack of the protease on the bait region of $\alpha_M$. (12) Second, when proteases are employed to activate and incorporate antigens into $\alpha_M$, the proteinase within $\alpha_M^*$ is active and can cleave co-incorporated proteins as noted above. Third, the presence of a co-incorporated proteinase within the $\alpha_M^*$-antigen complex competes with the nonproteolytic protein for space and binding sites within $\alpha_M^*$. This decreases the amount of antigen that can be incorporated (12). For all these reasons, the $\alpha_M^*$ used to prepare $\alpha_M^*$-antigen complexes should ideally be prepared by the reaction of $\alpha_M$ with NH$_3$. This preparation suffers from none of the disadvantages cited above. Moreover, this preparation can be made in advance and stored for long periods of time until required to produce an $\alpha_M^*$-antigen complex. Given the increased interest in employing $\alpha_M^*$ to target antigens for presentation, it is important to understand the chemistry of the reaction between nonproteolytic proteins and $\alpha_M$-NH$_2$ at elevated temperatures. To date, this reaction has not been characterized in any detail.

The current studies were undertaken to address these issues. It is demonstrated that the reaction rate is equally and proportionally dependent upon the concentrations of both the ligand and $\alpha_M^*$, indicating that the incorporation reaction is a mixed, bimolecular reaction occurring under second-order conditions. The absence of both a lag phase and a biphasic reaction suggests a lack of cooperativity among the four thiol esters. The finding that this reaction is bimolecular is consistent with the binding of HEL occurring through nucleophilic substitution. This is supported by the finding that nucleophilic attack is the mechanism by which proteases and small primary amines bind to $\alpha_M$ (4, 5, 21). The specific rate constant for HEL incorporation into $\alpha_M^*$ is significantly higher than the specific rate constants of nucleophilic attack by charged amines on native $\alpha_M$. Incorporation of HEL is ~64 times faster than thiol ester cleavage by ammonia and >1000 times faster than cleavage with isopropylamine (21). This may result from the effect of difference in thiol ester exposure or availability in the nascent state between these reactions. Nonproteolytic incorporation occurs by way of an exposed, reformed thiol ester; by contrast, primary amines are sterically hindered from reacting with the thiol ester in native $\alpha_M$, given its location in a hydrophobic pocket (4–6). Additionally, the large activation entropy of the reaction may contribute to the magnitude of the specific rate constant due to its effect on the free energy of the reaction.

The data for both pH and temperature also support nucleophilic substitution as the reaction mechanism. The increase in reaction rate as pH rises to 7.0 is consistent with an increase in reactivity of attacking nucleophiles, such as amines and sulfhydryls, as a consequence of deprotonation. Additionally, the

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**Table II**

| pH | $k$ (M$^{-1}$ s$^{-1}$) |
|----|-----------------------|
| 5.0 | 23.57 ± 2.13          |
| 6.0 | 39.01 ± 11.89         |
| 7.0 | 91.61 ± 17.84         |
| 8.0 | 83.25 ± 20.57         |
| 9.0 | 88.65 ± 17.78         |

$^{125}$I-HEL-$\alpha_M^*$ and $^{125}$I-HEL were brought to pH 5.0, 6.0, 7.0, 8.0, or 9.0 in 0.1 M PBS through buffer exchange. $^{125}$I-HEL binding was measured at 2.5, 5.0, 7.5, and 10.0 min. Additional details are given under "Experimental Procedures." Specific rate constants were calculated for each pH studied. The data represent the mean of three experiments ($\mu^2 > 0.98$).
The large activation energy is consistent with our initial studies on the binding of nonproteolytic ligands to α2M* (13) and reflects the thermodynamic unfavorability of thiol ester reformation. It has been observed that when α2M* is heated in the absence of a ligand, the thiol esters regenerate, and the molecule reverts to the native form (19). We hypothesize that heated α2M* passes through a state that mimics the proteolytically induced nascent state, such that nonproteolytic ligands can be incorporated. This is supported by the fact that during the conversion to α2M*, native α2M can only bind ligands while in the nascent state, in which the labile thiol esters are briefly exposed to ligands (4, 5).

Based on this data, we propose that the incorporation of ligands into α2M* occurs through a two-step reaction (Fig. 5) shown below.

\[
\begin{align*}
\alpha_2M^* \quad &\xrightarrow{k_1/4.1, \Delta} \quad (\alpha_2M) \\
(\alpha_2M) + \text{ligand} \; &\xrightarrow{k_2} \; \alpha_2M^*-\text{ligand}
\end{align*}
\]

(Eq. 5) (Eq. 6)

Our model incorporates a nascent-like state, <α2M>, that appears to be the transition state necessary for ligand binding. We propose that the thiol ester reforms but is then quickly subjected to nucleophilic attack by the ligand. Binding of the thiol ester, in the nascent-like state, to the ligand drives the formation of the α2M*-ligand complex instead of allowing the nascent-like state to proceed back to native α2M. The nascent state is considered to be a transient intermediate, and the inability to detect it when the reaction mixture is separated electrophoretically attests to its high reactivity. We suggest that only the first step of the reaction requires the addition of heat; in the nascent state, energy is not required for ligand binding, as shown by the ability of native α2M to react with and bind proteinases at 4 °C (1). This model addresses the reversibility of nascent-state formation shown by Gron et al. (19) and the irreversibility of ligand incorporation. This irreversibility may be due to stabilization of the ligand within the α2M* cage-like structure through protein-protein interactions. This proposed mechanism is consistent with the experimentally derived rate equation, with Step II as the rate-limiting reaction and the specific rate constant (k1) equal to \((k_1/k_2/k_{-1})\).

The Gron and Pizzo method of α2M*-antigen complex generation has been used by others to suggest that uptake by LRP/CD91 can result in antigen presentation on major histocompatibility complex class I (15). However, caution is required when interpreting this previous study. The α2M employed was obtained commercially, and the incubation of α2M with the antigen was performed for only 10 min at 50 °C. The form of α2M in the commercial preparation was not stated. It is our experience that commercial preparations show varying ratios of α2M: α2M*. In the extreme case, preparations consist predominately of one form or the other. Because native α2M cannot incorporate antigens by heating, the ratio of α2M:α2M* in such preparations is critical. Moreover, proteolytically activated forms of α2M* do not incorporate antigen. Thus, unless a commercial preparation of α2M:α2M* contains α2M-NH2, it will not be suitable for heat-induced generation of α2M*-antigen complexes. Assuming that the commercial preparations employed in these studies were predominately α2M*-NH2, the conditions under which α2M*-antigen complexes were produced are also critical. In the current study, we have derived equations that can be employed to calculate the amount of α2M*-antigen formed for any given time of incubation. The 10-min incubation
period employed in the above cited study is predicted to yield 0.8% $\alpha_2M^*$-antigen complex; however, only 0.1% complex production was reported (15). Optimal reaction conditions, as defined in the present study, will make it possible to obtain complex preparations in which every $\alpha_2M^*$ molecule carries multiple antigens. Such complexes can be expected to have significant effects on antigen presentation mediated by $\alpha_2M^*$.

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J. Biol. Chem. 2001, 276:41547-41552.
doi: 10.1074/jbc.M106357200 originally published online August 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106357200

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