Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1.

K Yanase, … , L Jarett, M P Madaio


A unique subset of anti-DNA antibodies enters living cells, interacts with DNase 1, and inhibits endonuclease activity, before their nuclear localization and subsequent attenuation of apoptosis. We now report that endocytosis of these immunoglobulins is mediated by cell surface binding to brush border myosin (myosin 1). Cellular entry and internalization via this unique receptor provides initial contact for entry and sorting these immunoglobulins to translocate to the nuclear pore and enter the nucleus, interact with DNase 1 within the cytoplasm, or recycle back to the cell surface. This internalization pathway provides clues to the translocation of large proteins across cell membranes and the functional effects of intracellular antibodies on cytopathology. This is the first demonstration that brush border myosin functions as a specific cell surface receptor for internalization of large proteins.
Receptor-mediated Cellular Entry of Nuclear Localizing Anti-DNA Antibodies via Myosin 1

Kumiko Yanase,* Robert M. Smith,‡ Antonio Puccetti,§ Leonard Jarett,¶ and Michael P. Madaio*  
*Penn Center for Molecular Studies of Kidney Diseases, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6144; ‡Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6144; and §Monoclonal Antibody Unit, Advanced Biotechnology Center and Department of Histology, University of Genova, Genova, Italy

Abstract

A unique subset of anti-DNA antibodies enters living cells, interacts with DNase 1, and inhibits endonuclease activity, before their nuclear localization and subsequent attenuation of apoptosis. We now report that endocytosis of these immunoglobulins is mediated by cell surface binding to brush border myosin (myosin 1). Cellular entry and internalization via this unique receptor provides initial contact for entry and sorting these immunoglobulins to translocate to the nuclear pore and enter the nucleus, interact with DNase 1 within the cytoplasm, or recycle back to the cell surface. This internalization pathway provides clues to the translocation of large proteins across cell membranes and the functional effects of intracellular antibodies on cytopathology. This is the first demonstration that brush border myosin functions as a specific cell surface receptor for internalization of large proteins. (J. Clin. Invest. 1997. 100:25–31.) Key words: autoimmunity • systemic lupus erythematosus • endocytosis • cell surface receptor • autoantibody

Introduction

Although cellular and nuclear localization of autoantibodies was initially described three decades ago, debate continues over both the validity and pathogenic relevance of the observation. It has been argued by some that it represents a fixation artifact, with movement of immunoglobulins into cells during tissue preparation (1, 2). Others have claimed that since an in vivo ANA is often found in both areas of inflammation and noninflamed tissues in autoimmune patients, the phenomenon does not have pathogenic relevance (2–4). By inference, therefore, if it is not pathogenic it is not worth studying. Nevertheless, we and others have found the observation intriguing and worthy of investigation, reasoning that the phenomenon may have more general biologic significance, whether or not it is pathogenically relevant (5–10).

In this regard, in the search for pathogenic lupus autoantibodies, we identified monoclonal anti-DNA antibodies, derived from lupus-prone mice, which crossed both the cell and nuclear membranes to localize within nuclei in living cells of multiple organs, after administration to normal mice (9, 11). Of potential pathologic significance, in the kidney this was associated with functional abnormalities, including glomerular hypercellularity and proteinuria (11). Subsequently, three MRL/lpr/lpr–derived autoantibodies with these properties were found to enter cultured cells and localize within nuclei (9). Initial cell surface binding was followed by distribution of immunoglobulins within the cytoplasm, subsequent clustering at the nuclear pore, and nuclear localization (9). Cellular uptake and nuclear localization of these immunoglobulins were dependent on their antigen binding regions, and structural analysis indicated that at least two of the three shared a conformational motif in the heavy chain CDR3 region resembling nuclear localization signals which direct nuclear import of proteins (12, 13). A suitable template was not available for modeling the other antibody although it also contained cationic amino acid residues within the CDRs resembling nuclear localization motifs (12).

The kinetics of both cellular uptake and nuclear entry of these antibodies were temperature dependent, suggesting that these processes were energy dependent and receptor mediated (9). The purpose of these studies was to more precisely define the events responsible for cellular entry and intracellular transit of this subset of anti-DNA antibodies.

Methods

Monoclonal antibodies, cell lines

The monoclonal anti-DNA antibodies H7, H72, and H9 were purified from hybridoma supernatants by affinity chromatography, as previously described (14, 15). The monoclonal antibody, MOPC-21 (mouse IgG1K; Sigma Chemical Co., St. Louis, MO) was used as a control. H35 rat hepatoma cells (obtained from R. Taub, University of Pennsylvania, Philadelphia, PA) were grown and maintained according to the originators (9).

Quantitation of nuclear–associated 125I-Ig in trypsin-treated cells

Previously described methods were used for labeling Ig (125I), coculture of cells with antibodies, and determination of the quantity of nuclear Ig (9). To determine if the interactive cell surface antigen was trypsin sensitive, H35 cells were grown in flasks to near confluence, harvested, and resuspended (107 cells/ml) in Krebs-Ringer Mops buffer containing 0.1% BSA. The cells were then incubated in the ab-

Received for publication 18 November 1996 and accepted in revised form 8 April 1997.

Address correspondence to Michael P. Madaio, M.D., Penn Center for Molecular Studies of Kidney Diseases, Department of Medicine, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, PA 19104-6144. Phone: 215-662-7627; FAX: 215-898-0189; E-mail: madaio@mail.med.upenn.edu

© The American Society for Clinical Investigation, Inc.

J. Clin. Invest. 0021-9738/97/0025-7 $2.00

Volume 100, Number 1, July 1997, 25–31
sence (control) or presence (trypsin-treated) of 1 mg/ml (1,130 BAEE U/ml) trypsin (type II, Sigma T-8128; Sigma Chemical Co.) for 15 min at 37°C, after which the trypsin was neutralized with 1 mg/ml trypsin inhibitor (type III-O, Sigma T-2011; Sigma Chemical Co.) in Krebs-Ringer Mops buffer at 4°C. Cell viability was monitored by trypan blue; viability was not affected by these procedures. After washing, the cells were incubated with 125I-Ig for 1 h at 37°C. Nuclear associated Ig was determined in nuclei isolated from the same cells, as previously described (9). The results are expressed as %I activity/mg nuclear protein (16).

**Evaluation of cell surface binding (FACS®)**

**Inhibition of cell surface binding using unlabeled antibody.** H7 was labeled with fluorescein using the Fluorescein-Ex protein labeling kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer’s instructions. The fluorescein-labeled antibodies maintained good activity as demonstrated by their capacity to localize within living cells (not shown). For inhibition studies, initially a titration curve was produced with fluorescein-labeled H7 (30 μg/ml) or MOPC-21 (30 μg/ml) for 1 h at 4°C. The cells were then washed with Krebs-Ringer Mops buffer containing 1% BSA, fixed in 1% paraformaldehyde/PBS (1 h, 4°C), and cell surface binding was determined by FACS® (Becton Dickinson, San Jose, CA).

**Inhibition of cell surface binding using trypsin.** Cells were either treated with trypsin or buffer (as described above) and then incubated with either H7 (30 mg/ml) or MOPC-21 (30 mg/ml) for 1 h at 4°C. After washing, the cells were incubated with fluorescein-labeled goat anti-mouse IgG for 1 h at 4°C, fixed in 1% paraformaldehyde/PBS and examined by FACS®.

**Monitoring cell surface Ig over time (FACS®).** The kinetics of Ig internalization and IgG recycling back to the cell surface were evaluated by determination of the quantity of cell surface IgG at varying intervals after coculture. For this purpose, 200 μl of H35 cells (10^7 cells/ml) was incubated with either H7 or MOPC-21 in Krebs-Ringer Mops buffer containing 1% BSA (triplicates) for 1 h at 4°C; at this temperature cell surface binding occurs; however, internalization does not (2). The cells were then washed once with Krebs-Ringer Mops buffer containing 1% BSA to remove unbound IgG (4°C), and then reincubated in Krebs-Ringer Mops buffer containing 1% BSA (without additional IgG) at either 37 or 4°C. At varying intervals, aliquots of cells were washed and then fixed in 1% paraformaldehyde/ PBS (1 h, 4°C). To determine the quantity of cell surface IgG, the aliquots were incubated with FITC-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), washed (PBS), fixed again (1% paraformaldehyde/PBS), and evaluated by FACS®.

**Simultaneous evaluation of cell surface binding and nuclear localization of antibodies by confocal immunofluorescence microscopy**

To monitor intracellular movements of IgG and cell surface IgG simultaneously in the same, nonpermeabilized cells, confocal microscopy was used to visualize both rhodamine-labeled H7 (by direct visualization) and cell surface H7 (by indirect immunofluorescence, using FITC labeled anti-mouse IgG). For this purpose, individual IgG were labeled with rhodamine in the following manner: 1.5 ml H7 (1 mg/ml) or MOPC-21 (1 mg/ml) were reduced with 0.1 M 2-mercaptoethanol for 90 min at 37°C and then incubated with 5 μl of Rhodamine X iodoacetamide (10 mg/ml in DMSO; Molecular Probes) overnight at 4°C. Unbound rhodamine was then removed by centrifugation/filtration using Centricron 30 (Amicon, Inc., Beverly, MA).

To monitor intracellular movements of IgG and to distinguish between cell surface and intracellular IgG, aliquots of rat hepatoma cells were incubated with rhodamine-labeled IgG (30 μg/ml) at 4°C. After 1 h the cells were washed with Krebs-Ringer Mops buffer containing 1% BSA to remove unbound IgG, and the cells were then reincubated in Krebs-Ringer Mops buffer containing 1% BSA (without additional IgG) at 37°C. After varying intervals, the cells were washed with PBS and fixed in 1% paraformaldehyde/PBS (1 h, 4°C). To detect cell surface IgG, the cells were washed again with PBS and stained with FITC-labeled goat anti-mouse IgG. The cells were intentionally not permeabilized during the 1% paraformaldehyde fixation, so that the FITC-labeled, goat anti-mouse IgG only detected surface bound IgG. Using confocal microscopy, the location of rhodamine-labeled H7 and cell surface IgG (FITC) was visualized at the appropriate wavelength in the same cells.

**Immunoprecipitation of interactive cell surface proteins**

The surface proteins of H35 rat hepatoma cells (10^7 cells/ml) were biotinylated by incubation with NHS-biotin (0.5 mg/ml; Pierce, Rockford, IL) in labeling buffer (135 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM MgSO4,7H2O, 1 mM CaCl2, 6 mM NaHCO3) at 4°C for 20 min. The reaction was quenched with 15 mM glycine in labeling buffer at 4°C, and the cells were then resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM PMSF, 0.5% NP-40, 0.02% NaN3, 0.5% Triton X-100) and lysed. The cell lysates were centrifuged (1,000 g) for 15 min to remove nuclei. The supernatants were incubated with either H7 (30 μg/ml) or MOPC-21 (30 μg/ml) overnight at 4°C. IgG complexes were precipitated with protein-A/G-agarose for 1 h at 4°C. The immunoprecipitates were prepared in Laemmli sample buffer containing 50 mM DTT. The samples were resolved by SDS-polyacrylamide gel (7.5%) electrophoresis and transferred to Immobilon-P transfer membrane (Millipore Co., Bedford, MA). The membrane was then incubated with horseradish peroxidase–linked streptavidin (Sigma Chemical Co.) at a dilution of 1:2,000 for 1 h.

In separate experiments, after resolution by SDS-PAGE and transfer to Immobilon-P transfer membrane, the protein blots were incubated with mouse anti–brush border myosin 1 antibody (1:1,000; Chemicon International Inc., Temecula, CA). After 2 h, the blots were washed and then incubated with horseradish peroxidase–linked anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN). The appropriate size band was cut out. Multiple samples were sent for microsequence analysis to two laboratories who performed independent analysis and provided independent sequence analysis of internal peptides (Harvard Microchem, Cambridge, MA, and Protein Chemistry Laboratory, Cancer and Diabetes Centers at University of Pennsylvania, Philadelphia, PA).

**Amino acid sequence analysis of immunoprecipitated cell surface protein**

The immunoprecipitated cell surface protein was transferred to Immobilon-P transfer membrane as described above. After Ponceau-S staining, the appropriate size band was cut out. Multiple samples were sent for microsequence analysis to two laboratories who performed independent analysis and provided independent sequence analysis of internal peptides (Harvard Microchem, Cambridge, MA, and Protein Chemistry Laboratory, Cancer and Diabetes Centers at University of Pennsylvania, Philadelphia, PA).

**Results**

Specific cell surface events mediating cellular entry of these immunoglobulins were suggested by previous findings that internalization and nuclear uptake of radiolabeled antibodies were specifically and significantly inhibited by unlabeled nuclear localizing antibodies but not by other anti-DNA antibodies which did not enter cells (9). Furthermore, the nuclear localizing anti-DNA antibodies bound to cell surfaces by FACS®, whereas other monoclonal anti-DNA antibodies which produced neither intranuclear nor extracellular immune deposits did not (data not shown). Specific cell surface binding by IgG was confirmed by short-term culture experiments using
fluorescein-labeled H7 and H35 cells. Unlabeled H7 significantly inhibited cell surface binding of fluorescein-H7 in a dose-dependent manner, whereas MOPC-21 did not inhibit binding (Fig. 1). Additionally preincubation of cells with trypsin before coculture with nuclear localizing antibodies significantly inhibited both cellular and nuclear accumulation of the immunoglobulins (Fig. 2, a and b), suggesting that internalization is mediated by a cell surface protein.

To address the possibility of a specific receptor on the plasma membrane, the capacity of the anti-DNA antibodies to immunoprecipitate cell surface proteins was evaluated. For this purpose, H35 hepatoma cell surfaces were biotinylated and incubated with the nuclear localizing anti-DNA antibody H7. After immunoprecipitation with protein A/G, the antibody–cell surface protein complexes were separated and visualized. As illustrated in Fig. 3, a single 110-kD cell surface protein was precipitated by the nuclear localizing anti-DNA antibody H7. The other nuclear localizing antibodies (H9 and H72) also reacted with this protein (not shown), whereas MOPC-21 never immunoprecipitated cell surface proteins.

Using identical methodologies, the 110-kD protein was isolated. Three separate preparations were submitted for amino acid sequence analysis to two different laboratories. Five independently derived peptides from the 110-kD protein are illus-
trated in Fig. 4. As shown, there was 100% homology with myosin 1 (brush border myosin 1) (17).

Anti–brush border myosin 1 antibodies reacted with the cell surface protein immunoprecipitated by the nuclear localizing anti-DNA antibodies (Western blotting) confirming the identity of the protein (Fig. 5). However, the anti–brush border myosin 1 antibodies neither bound to cell surfaces nor were internalized in living cells, although they bound to cell membranes of lightly permeabilized and fixed cells (data not shown). This difference in behavior suggests that the epitopes recognized by these antibodies are different, and that the epitope recognized by the nuclear localizing anti-DNA antibodies is contained within an extracellular domain of myosin 1 which facilitates internalization.

To more precisely characterize the surface events and transist of the immunoglobulins through the cell, cell surface and intracellular immunoglobulins were monitored simultaneously in the same cells. For this purpose, confocal microscopy was used to analyze the movement of rhodamine-labeled nuclear localizing antibody H7 intracellularly in living cells; simultaneously, surface bound immunoglobulin was assessed by indirect immunofluorescence. (The fluoresceinated anti–mouse immunoglobulins did not enter the nonpermeabilized cells; they only detected surface immunoglobulins.) In parallel, cell surface binding was monitored by FACS®.

The experimental protocol involved initial incubation of immunoglobulins (either nuclear localizing antibodies or control immunoglobulins) with cells for 1 h at 4°C, so that there was cell surface binding, but intracellular translocation of immunoglobulins was prevented (9). The cells were then washed to remove unbound immunoglobulins, and medium (without immunoglobulins) was added back to the cultures. The cells were then warmed to 37°C to initiate internalization of the bound immunoglobulins, and they were maintained at 37°C for 4 h. Aliquots of cells were analyzed throughout the study period. The results are illustrated in Figs. 6 and 7.

As expected, at 4°C cell surface binding of nuclear localizing antibodies occurred rapidly, and internalization of immunoglobulins was not observed. Upon removing unbound immunoglobulins from the medium and warming the cells to 37°C, the surface bound antibodies rapidly entered cells (Figs. 6 and 7). Once internalized, the intracellular antibodies traf-
flicked in two patterns: into the nucleus and back to the cell surface. These pathways were convincingly demonstrated by FACS® analysis (Fig. 6) and confocal microscopy (Fig. 7). As shown, nuclear uptake of immunoglobulins increased throughout the study period (Fig. 7). By comparison, cell surface immunoglobulins decreased immediately after warming, reaching a nadir at 15 min (Figs. 6 and 7); this decline in surface immunoglobulins was associated with the increase in intracellular immunoglobulin just described. Over the next 2 h, however, an increase of surface immunoglobulin was also consistently observed (Figs. 6 and 7). Since unbound antibodies were removed from the medium before rewarming, we interpret this...
observation to indicate recycling of intracellular immunoglobulins back to the cell surface. By contrast, when the temperature was held constant at 4°C throughout the entire study period, there was a gradual reduction in surface-bound immunoglobulin (Fig. 6), most likely due to dissociation of immunoglobulin from the cell membrane into the medium.

Discussion

The results demonstrate that myosin 1 functions as a specific cell surface receptor for internalization of nuclear localizing anti-DNA antibodies. The identity of this receptor was convincingly demonstrated using several approaches, including immunoprecipitation of the appropriate size protein from the cell surface; complete (100%) sequence homology of five peptides derived from the immunoprecipitated cell surface protein with myosin 1; and recognition of the immunoprecipitated cell surface protein by anti–myosin 1 antibodies. Supporting evidence is also provided by the observation that the immunoprecipitates contained calmodulin (our unpublished observation), which is known to complex with myosin 1. Collectively, these observations provide novel insights into both the internalization of large proteins into cells and the modulation of inflammation during systemic autoimmunity.

Internalization of nuclear localizing antibodies after interaction with myosin 1 is consistent with recent evidence regarding the role of this cell surface receptor (18–20). Although the function of this class of receptors is not completely understood, myosin 1 is expressed in a wide range of tissues (20). Based on its structure and cellular localization, it has been implicated in vesicle-mediated solute transport, and recent observations of MYO3 and MYOS deletion mutants in yeast support this viewpoint (19). Our previous finding that internalized antibodies were found in vesicles within the cytoplasm is consistent with this notion, although internalized immunoglobulins were also observed in other intracellular locations (9). Nevertheless, the observation that the nuclear localizing autoantibodies were present in the nuclei of multiple organs in vivo (11) is consistent with entry via a ubiquitous receptor like myosin 1.

The cellular entry of the nuclear localizing anti-DNA antibodies via myosin 1 is particularly intriguing in light of our recent findings on the intracytoplasmic activity of these immunoglobulins (21). Once internalized, the nuclear localizing antibodies reacted with DNase 1 within the cytoplasm and inhibited DNase 1 enzymatic activity in vivo. Furthermore, preincubation of cells with nuclear localizing anti-DNA antibodies, before challenge with apoptotic stimuli, blocked features of apoptosis. Taken together with the present findings, it is tempting to speculate that the intracytoplasmic anti-DNA antibody–DNase 1 interaction is facilitated by the close association of myosin 1 with DNase 1 in this location (22). Of potential clinical relevance in the context of ongoing inflammation, the anti-DNA antibody–DNase 1 interaction could have dire effects, especially in situations where apoptosis is essential for recovery (23).

Whether the nuclear localizing anti-DNA antibodies dissociate from myosin 1 (or DNase 1) within the cytoplasm before nuclear entry or move into the nucleus as an antigen–antibody complex requires further study. As previously mentioned, conformational analysis of the antibodies indicate that their antigen binding regions share a tertiary structure resembling nucleolocalization sequences (12), although the requirement for accessory molecules (i.e., myosin 1 or DNase 1) needs to be determined. Nevertheless, from the present studies, it also appears that a fraction of the internalized antibodies is recycled back to the cell surface. Whether these recycled immunoglobulins originate from the nucleus or are short-circuited through a different cytoplasmic pool will be addressed in future experiments. However, reexpression of immunoglobulins on the cell surface (Figs. 6 and 7), especially if altered during recycling, could serve as a neoantigen for either immunoglobulin deposition or T-cell activation, with subsequent augmentation of the inflammatory response.

In any event, the intracellular fate of the anti-DNA antibodies is likely due to the antigen binding properties of the internalized immunoglobulins. This functional characteristic most likely contributes to the divergent behavior of internalized autoantibodies previously reported by others, including Alarcon-Segovia, Reichlin, and others (24–31). Once internalized, the autoantibodies move to the location of their target ligand. This interpretation is also consistent with recent descriptions of the intracellular behavior of “intrabodies.” These Fv or Fab antibody fragments, synthesized within the cell, contain additional peptides on the 5′ region of the heavy chain which facilitate the movement of the fragments to a specific subcellular location, defined by their leader peptides (32). Once at the desired location, the antibody fragments can then bind to the desired ligand. In some circumstances, these intrabodies have been observed to also alter cellular functions associated with their target antigen, in a manner similar to that observed with the antiapoptotic activity of the nuclear localizing anti-DNA antibodies (21).

Further study of the intracellular events facilitating internalization and intracellular movement of the nuclear localization of anti-DNA antibodies should therefore provide insight into the movement of large proteins across cell membranes. The promiscuous behavior of these nuclear localizing anti-DNA antibodies may also provide the potential means to move large molecules across cellular membranes into and within the cell.

Acknowledgments

We thank Dr. Marina Fabbri for advice and discussion of the manuscript, and Julie Lin for technical assistance.

This work was supported by a George M. O’Brien Kidney and Urological Research Center Grant (DK45191), and the following individual Public Health Service Awards: DK 33694, AI 27915 (M.P. Madaio), DK 28143 (L. Jarett), and the DCI RED FUND.

References


Cellular Entry of Autoantibodies via Myosin 1