Human natural resistance-associated macrophage protein is a new type of microtubule-associated protein

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Received 16 February 1998; revised version received 17 April 1998

Abstract Natural resistance-associated macrophage protein 1 (NRAMP1) is a putative membrane protein that dominates natural resistance to infection. An NRAMP1-glutathione S-transferase fusion protein was used to test the ability of the NRAMP1 NH_2-terminal domain to bind to taxol-stabilized microtubules. Co-sedimentation analysis showed that the fusion protein binds to microtubules. Although the NH_2-terminal domain of the NRAMP1 molecule has structural homology with the Pro-rich region of microtubule-associated protein 4 (MAP4), the presence of the MAP4 microtubule-binding domain fragment had little effect on the binding of the fusion protein to microtubules.

Key words: MAP; Microtubule; Natural resistance; NRAMP1

1. Introduction

Natural resistance, as well as the susceptibility of a host to infection by intracellular pathogens, is controlled by a single dominant gene locus, *Lsh*/*Ity*/*Bcg* [1–3]. Vidal et al. [4] isolated a candidate gene for *Bcg* from a mouse pre-B cell cDNA library, and designated it as the 'natural resistance-associated macrophage protein gene' (*Nramp*). Several *Nramp* genes have been cloned [5–7] and have been shown to encode a membrane protein with 10–12 putative transmembrane domains. This protein is considered to direct the capacity of host macrophages to resist the intracellular replication of ingested parasites, such as *Mycobacterium*. We isolated a human *NRAMP1* cDNA from a human monocyte cDNA library [6], and showed that human NRAMP1 was expressed in established hematopoietic cell lines (monocyte/macrophages and B- and T-lymphocytes) and human peripheral blood leukocytes (mainly in monocytes) [8,9]. The NH_2-terminal region of human NRAMP1 has structural homology with the SH3-binding motif and the microtubule-associated protein 4 (MAP4) [10].

MAP4 is a non-neural MAP, which has a microtubule-binding domain similar to that of the neural MAPs, MAP2, and tau [11–13]. Human NRAMP1 has structural homology with the Pro-rich region [10], one of the three subdomains of the MAP4 microtubule-binding domain [11]. We [14] and others [15–18] have prepared and characterized several recombinant polypeptides of the MAP4 microtubule-binding domain.

The function of NRAMP1 is unknown at present. Immunohistochemical analysis provided evidence that human NRAMP1 is located and distributed on the plasma membrane [10]. In addition to the structural homology to MAP4, we found that α- and β-tubulins are the only molecules that were able to associate with the NH_2-terminal cytoplasmic domain of the human NRAMP1 molecule in a human lymphoma cell extract [10]. The lines of evidence suggested that human NRAMP1 functions as a linker molecule between phagocytotic vesicles and microtubules [10]. However, there is no direct evidence that human NRAMP1 binds to microtubules, which are polymers of α- and β-tubulin heterodimers.

Previously, we prepared the GST-NRAMP1$^{1\sim81}$ protein, a fusion protein of glutathione S-transferase (GST) and the NH_2-terminal 81 residues of the human NRAMP1 molecule, to study the in vitro activity of the NH_2-terminal cytoplasmic domain of the NRAMP1 molecule [10]. In this paper, we examined the interaction between the fusion protein and taxol-stabilized microtubules in vitro. The ability of the fusion protein to compete with MAP4 for microtubule binding was also tested, using the PA_4 fragment, a recombinant peptide corresponding to the microtubule-binding domain of MAP4.

2. Materials and methods

2.1. Materials

Taxol was supplied by Dr. N. Lomax, of the Natural Products Branch, National Cancer Institute, Bethesda, MD. It was dissolved in dimethyl sulfoxide to a stock concentration of 1 mM and was stored at −20°C. The PA_4 fragment expression plasmid was supplied by Dr. Murofushi (University of Tokyo). All other materials were of reagent grade.

2.2. Protein preparations

The GST and GST-NRAMP1$^{1\sim81}$ were prepared as described [10]. The PA_4 fragment was prepared according to Aizawa et al. [15]. Native MAP4 was purified from bovine adrenal cortex by the method of Murofushi et al. [19]. Fig. 1 illustrates the schematic structures of the proteins used in this study, along with a putative structure of the full-length human NRAMP1.

Bovine brain tubulin was purified from two-cycled microtubule protein fractions [20] by the method of Weingarten et al. [21]. The protein concentration was determined by the method of Lowry et al. [22] using bovine albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [23], using a 12.5% gel. The gels were stained with Coomassie brilliant blue R250.

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Abbreviations: NRAMP1, Natural resistance-associated macrophage protein 1; MAP, microtubule-associated protein; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST extract, *E. coli* crude extracts containing GST; GST-NRAMP1$^{1\sim81}$ extract, *E. coli* crude extracts containing GST-NRAMP1$^{1\sim81}$

PII S0014-5793(98)00488-8

2.3. Co-sedimentation analysis with taxol-induced microtubules

Escherichia coli crude extracts, containing either GST (GST extract) or GST-NRAMP1$^{1\sim81}$ (GST-NRAMP1$^{1\sim81}$ extract), were prepared from *E. coli* JM109 harboring each expression plasmid, according to Kishi et al. [10]. Each extract (22 μl) was mixed with purified tubulin (final concentration, 15 μM) in 100 MEM buffer (100 mM 2-morpholinoethanesulfonic acid, 0.5 mM $MgSO_4$, 0.1 mM EGTA, 0.5 mM GTP, pH 6.8) containing 30 μM taxol (final volume, 50 μl). The mixtures were incubated at 37°C for 30 min, and were centrifuged at 15000×*g* for 30 min at 30°C. The precipitates were resuspended in 50 μl of an alkaline solution (0.2 M Na_2CO_3, 0.1 M NaOH), and the supernatants and the precipitates were subjected to SDS-PAGE. The two-dimensional band size of the GST-NRAMP1$^{1\sim81}$ on the polyacrylamide gels was measured by an imaging analyzer (scanner: ScanJet 4C, HEWLETT PACKARD, software: NIH image).

2.4. Co-sedimentation analysis with PA_4 fragment-induced microtubules

GST-NRAMP1$^{1\sim81}$ extract was mixed with purified tubulin (final concentration, 15 μM) in 100 MEM buffer containing 10 μM PA_4 fragment. The mixture was incubated at 37°C for 10 min, and was centrifuged at 15000×*g* for 10 min at 30°C. The precipitate was resuspended in the 100 MEM buffer at 0°C, and was centrifuged at 15000×*g* at 4°C for 10 min. And further, the supernatant was incubated at 37°C for 10 min, and was centrifuged at 15000×*g* at 30°C for 10 min. The supernatants and the precipitates of each centrifugation step were subjected to SDS-PAGE.

2.5. Competition analysis

Various concentrations of the PA_4 fragment were mixed with purified tubulin (final concentration, 15 μM) in phosphate buffer (10 mM sodium phosphate, 6 mM $MgSO_4$, 0.5 mM GTP, pH 6.8) containing 30 μM taxol (final volume, 50 μl). Microtubules were allowed to polymerize by an incubation at 37°C for 15 min. Then, either MAP4 or the GST-NRAMP1$^{1\sim81}$ (final concentration, 5 μM) plus glutathione (final concentration, 50 mM) was added to the mixtures, and then the mixtures were incubated for an additional 30 min at 37°C. After centrifugation at 15000×*g* at 30°C for 30 min, the precipitates were resuspended in 50 μl of an alkaline solution, and subjected to SDS-PAGE. The two-dimensional band sizes of the GST-NRAMP1$^{1\sim81}$, MAP4, and the PA_4 fragment on the polyacrylamide gels were measured by an imaging analyzer, as described above.

3. Results

3.1. Binding of GST-NRAMP1$^{1\sim81}$ to taxol-stabilized microtubules

To investigate the possibility of an NRAMP1-microtubule interaction, we first tested whether the GST-NRAMP1$^{1\sim81}$ co-sediments in vitro with taxol-stabilized microtubules, using the *E. coli* crude extract containing GST-NRAMP1$^{1\sim81}$ (Fig. 2).

Tubulin polymerizes in the presence of taxol, and the reconstituted microtubules can be precipitated by centrifugation (Fig. 2, lane 1). If the GST-NRAMP1$^{1\sim81}$ binds to microtubules, then GST-NRAMP1$^{1\sim81}$ would co-precipitate with the microtubules under these conditions. Lanes 2 and 3 are the control studies: Under the same conditions as in lane 1, the GST alone was recovered in the supernatant, as was the GST-NRAMP1$^{1\sim81}$. Trace amounts of both proteins were observed in the precipitates, suggesting that the GST and the GST-NRAMP1$^{1\sim81}$ are labile against the warming process (37°C, 30 min). When each extract was mixed with microtubules, about half of the added GST-NRAMP1$^{1\sim81}$ co-sedimented with the microtubules (lane 5), while most of the GST was recovered in the supernatant (lane 4). This indicated that the GST-NRAMP1$^{1\sim81}$ has an activity to bind to microtubules in vitro, and that the NRAMP1 portion in the fusion protein is responsible for the binding activity.

The quantitation by imaging analyzer revealed that the concentration of added GST-NRAMP1$^{1\sim81}$ was 10 μM and that of the bound GST-NRAMP1$^{1\sim81}$ was 4 μM. The binding stoichiometry was 0.3 mol of GST-NRAMP1$^{1\sim81}$/mol of tubulin dimer. In the same condition, 7 μM of the PA_4 fragment bound to microtubules and the stoichiometry was 0.5 mol of PA_4 fragment/mol of tubulin dimer (data not shown). The difference in the apparent stoichiometry is most likely due to the difference in the binding affinities, namely, the binding affinity of the GST-NRAMP1$^{1\sim81}$ is smaller than that of the PA_4 fragment.

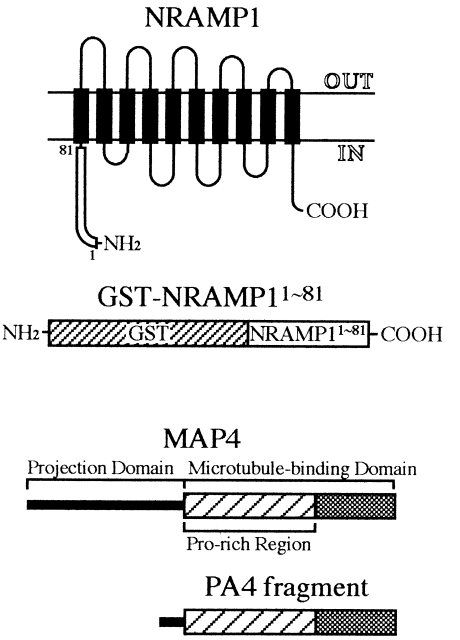

Fig. 1. Schematic representation of the putative structure of the NRAMP1, the GST fusion protein GST-NRAMP1$^{1\sim81}$, MAP4, and the PA_4 fragment.

3.2. Purification of the GST-NRAMP1$^{1\sim81}$

To analyze the details of the NRAMP1-microtubule interaction, the GST-NRAMP1$^{1\sim81}$ was purified using glutathione Sepharose 4B (Pharmacia Biotech), according to the supplier's manual. We attempted to digest the purified GST-NRAMP1$^{1\sim81}$ with Factor Xa protease to remove the GST portion of the fusion protein. However, the purified GST-NRAMP1$^{1\sim81}$ became insoluble instantly under the digestion conditions used in this study (50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5), and appropriate digestion conditions were not found. In addition, the purified GST-NRAMP1$^{1\sim81}$ was extremely unstable in the Good's buffer system, which is commonly used for in vitro microtubule reassembly analysis [24].

The purified GST-NRAMP1$^{1\sim81}$ was found to be stable in phosphate buffer in the presence of 50 mM reduced glutathione. This condition was inappropriate for the digestion with Factor Xa protease, but it is worth noting that phosphate buffer is used by several groups for microtubule assembly experiments [25,26]. We also confirmed, by electron microscopy, that the structure of the microtubules was normal in the phosphate buffer system used in this study (data not shown). In the following experiment, we tested the microtubule-binding activity of the purified GST-NRAMP1$^{1\sim81}$ using the phosphate buffer system.

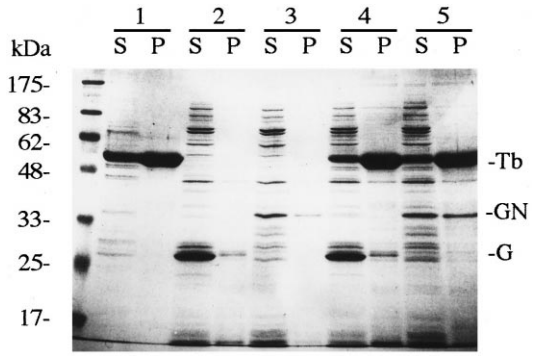

Fig. 2. Co-sedimentation analysis of the GST-NRAMP1$^{1\sim81}$ with taxol-induced microtubules. Each sample was centrifuged, and then the supernatants (S) and the precipitations (P) were subjected to SDS-PAGE. Lane 1, tubulin alone; lane 2, GST extract alone; lane 3, GST-NRAMP1$^{1\sim81}$ extract alone; lane 4, GST extract mixed with microtubules; lane 5, GST-NRAMP1$^{1\sim81}$ extract mixed with microtubules. The numbers on the left side indicate the positions of the marker proteins: fusion of *E. coli* maltose-binding protein (MBP) and *E. coli* β-galactosidase (175 kDa), fusion of MBP and paramyosin (83 kDa), bovine glutamic dehydrogenase (62 kDa), rabbit aldolase (48 kDa), rabbit triosephosphate isomerase (33 kDa), bovine β-lactoglobulin A (25 kDa), and chicken lysozyme (17 kDa). Tb, GN, and G show the positions of tubulin, GST-NRAMP1$^{1\sim81}$, and GST, respectively.

3.3. *Competition analysis of GST-NRAMP1$^{1\sim81}$ and PA$_4$ fragment for binding to microtubules*

By measuring the amounts of MAPs that co-sedimentated with the microtubules, one can analyze the details of the MAP-microtubule interaction. We have determined the dissociation constant and the binding stoichiometry of MAP4-truncated fragments to microtubules (Tokuraku, K., Katsuki, M., Nakagawa, H. and Kotani, S., manuscript in preparation). However, these biochemical properties are usually determined in the Good's buffer system. So, even if we determined these properties of the GST-NRAMP1$^{1\sim81}$ in the phosphate buffer, they would not be comparable with those of other microtubule-associated proteins, because of the different buffer systems. Therefore, the competition between GST-NRAMP1$^{1\sim81}$ and other known MAPs was analyzed instead.

The PA$_4$ fragment is a truncated fragment of MAP4, and contains its microtubule-binding domain (Fig. 1). The binding of the PA$_4$ fragment to microtubules was analyzed, and revealed that this fragment still has full microtubule-binding activity (Tokuraku, K., Katsuki, M., Nakagawa, H. and Kotani, S., manuscript in preparation). The activity of the PA$_4$ fragment in the phosphate buffer was also examined, and the fragment was found to co-sediment with the microtubules in the same manner as with the Good's buffer system, although the amount that sedimented was slightly decreased (data not shown).

When the PA$_4$ fragment was added to either the MAP4-microtubule mixture or the GST-NRAMP1$^{1\sim81}$-microtubule mixture, the amount of the PA$_4$ fragment that bound to the microtubules increased with increasing concentrations of the added fragment, and the binding was saturated at about 10 μM (Fig. 3a and b, closed circles). The binding of intact MAP4 decreased as the concentration of the PA$_4$ fragment increased (Fig. 3a, open circles), indicating that the PA$_4$ fragment competed with MAP4 for binding sites on the microtubules. On the other hand, the binding of the GST-NRAMP1$^{1\sim81}$ to the microtubules remained unaffected throughout the examined concentration range (Fig. 3b, squares). The binding of PA$_4$ fragment was also unaffected by the presence of GST-NRAMP1$^{1\sim81}$: 100% binding of the PA$_4$ fragment could be obtained without affecting the binding of the GST-NRAMP1$^{1\sim81}$ (Fig. 3b).

3.4. *Binding of GST-NRAMP1$^{1\sim81}$ to PA$_4$ fragment-induced microtubules*

We then examined whether the GST-NRAMP1$^{1\sim81}$ co-exists with the PA$_4$ fragment-induced microtubules through assembly cycles, since there is no competition between the PA$_4$ fragment and the GST-NRAMP1$^{1\sim81}$ (Fig. 3).

The GST-NRAMP1$^{1\sim81}$ extract was mixed with purified tubulin and the PA$_4$ fragment, and the microtubule protein fraction was prepared by the assembly-disassembly cycles (Fig. 4). Lanes 1 and 2 are supernatants and precipitates of the first assembly cycle. The GST-NRAMP1$^{1\sim81}$ co-sedimentated with the microtubules (lane 1) and a large quantity of the GST-NRAMP1$^{1\sim81}$ was recovered in the supernatant after cold treatment (lane 2), indicating that the GST-NRAMP1$^{1\sim81}$ cycles with microtubule proteins. Lane 3 are the supernatant and the precipitate after the second assembly step. In this step, the GST-NRAMP1$^{1\sim81}$ was the only protein in the *E. coli* extract that co-sedimentated with microtubules. All the other visible bands were the degradation products of the PA$_4$ fragment, as revealed by immuno-blotting experiments (data not shown). Because of the proteolytic

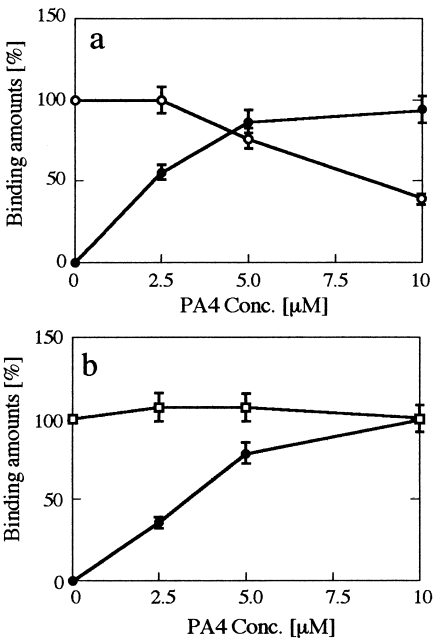

Fig. 3. Competition analysis of GST-NRAMP1$^{1\sim81}$ and the PA$_4$ fragment for binding to microtubules. Various concentrations of the PA$_4$ fragment and a constant concentration (5 μM) of either MAP4 (a) or GST-NRAMP1$^{1\sim81}$ (b) were mixed with microtubules. The mixtures were centrifuged, and then the precipitations were subjected to SDS-PAGE. The amounts of sedimentated MAP4 (open circles), GST-NRAMP1$^{1\sim81}$ (squares), and the PA$_4$ fragment (closed circles) are plotted against the amount of added PA$_4$ fragment. '100%' in the ordinate represents the binding amounts of the PA$_4$ fragment (10 μM) alone, MAP4 (5 μM) alone, or GST-NRAMP1$^{1\sim81}$ (5 μM) alone.

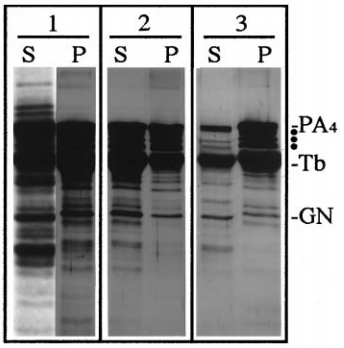

Fig. 4. Co-sedimentation analysis of the GST-NRAMP1$^{1\sim81}$ with PA_4 fragment-induced microtubules. Lanes 1 and 2 are the assembly step and the disassembly step, respectively, of the first cycle. Lane 3 is the assembly step of the second cycle. S and P indicate the supernatants and the precipitates of each step. PA_4, Tb and GN show the positions of PA_4 fragment, tubulin and GST-NRAMP1$^{1\sim81}$, respectively. The dots show the main degradation products of the PA_4 fragment.

activity in the *E. coli* extract, the cycling experiment could not be furthered.

4. Discussion

In this paper, we demonstrated that the GST-NRAMP1$^{1\sim81}$ has the microtubule-binding activity in vitro. Competition analysis indicated that the GST-NRAMP1$^{1\sim81}$ could not compete with PA_4 fragment for binding to microtubules in this assay system. Since it was demonstrated here that the cytoplasmic domain of human NRAMP1 binds to microtubules, the hypothesis that NRAMP1 mediates between endosomes and microtubules [10] was confirmed.

The MAP2, MAP4, and tau proteins share a homologous sequence in their carboxyl-terminal basic domains [11], and these MAPs are considered to interact with microtubules in a similar fashion. In fact, MAP2 and tau, but not MAP1, compete with the PA_4 fragment for microtubule binding (Kuroya, T., Tokuraku, K., Katsuki, M. and Kotani, S., manuscript in preparation). Competition between tau and the microtubule-binding domain fragment of MAP2 has also been reported [27]. The NH_2-terminal cytoplasmic domain of human NRAMP1 has an amino acid sequence homologous to the Pro-rich region in MAP4 [10], yet our data show that GST-NRAMP1$^{1\sim81}$ did not compete with the PA_4 fragment, indicating that the microtubule-binding mechanism of NRAMP1 is different not only from that of MAP4, but also from those of MAP2 and tau.

Among the three subdomains (the AP sequence region, the Pro-rich region, and the tail region) in the microtubule-binding domains of MAP2, MAP4 and tau, the most homologous region is the AP sequence region [11]. This region is essential to promote tubulin polymerization and to bind to microtubule [15], and binding site in tubulin has been revealed [28,29]. Although the Pro-rich region of MAP2, MAP4 and tau have similar amino acid compositions, significant sequence homology is not observed among the Pro-rich region of the three MAPs [11]. Our result, which shows that the NRAMP1 and MAP4 do not compete in spite of the sequence homology, supports the idea that the Pro-rich region does not play a dominant role in determining the binding specificity.

Other well-known microtubule-associated proteins are MAP1 and the microtubule-dependent motor proteins [30,31], but the NH_2-terminal cytoplasmic domain of NRAMP1 has no structural homology with these proteins [10]. The motor proteins contain the ATP-binding domain in addition to the microtubule-binding domain. NRAMP1 does not contain the so-called ATP-binding cassette [10], and we confirmed that ATP had no effect on the binding of GST-NRAMP1$^{1\sim81}$ to microtubules (data not shown). Consequently, NRAMP1 is neither a MAP1-like MAP nor a motor protein. Further, while all of the known MAPs are soluble proteins, NRAMP1 is a polytrophic integral membrane protein with structural features common to prokaryotic and eukaryotic transporter proteins [10]. These lines of evidence indicate that NRAMP1 is a new microtubule-associated protein with unique characteristics. It may form a new category of MAPs.

Intracellular pathogens are taken up in phagocytic macrophages, and are killed after phagolysosomal fusion, which is assumed to be controlled by a single dominant gene locus, designated *Lsh/Ity/Bcg*. Since the candidate gene product of *Bcg*, NRAMP1, shows microtubule-binding activity, this protein could mediate microtubule-dependent phagosome and/or lysosome transport. Whether the motor activity resides in NRAMP1 itself or in another associated protein(s) remains to be solved. Recently, in vivo phosphorylation of NRAMP1 was demonstrated [32]. Studies of the phosphorylation-dependent regulation of the NRAMP1-microtubule interaction are now in progress.

Acknowledgements: We wish to thank Dr. Murofushi for providing the expression plasmids for the PA_4 fragment, and Dr. N. Lomax, of the Natural Products Branch, National Cancer Institute, for providing the taxol. Thanks are also due to Dr. Brenda Guthrie (SKYBAY Scientific Editing) for reading the manuscript.

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