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One-step separation of myristoylated and nonmyristoylated retroviral matrix proteins

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ABSTRACT

N-terminal myristoylation of retroviral matrix proteins is essential for the targeting of the Gag polyproteins to the plasma membrane. To investigate the effect of the myristoylation on the structure and membrane binding ability of the matrix proteins, it is necessary to prepare their myristoylated forms. We present purification of myristoylated matrix proteins of the mouse mammary tumor virus and murine leukemia virus, two morphogenetically distinct retroviruses. The proteins were expressed in *Escherichia coli* coexpressing a yeast N-myristoyltransferase. This *E. coli* expression system yielded a mixture of myristoylated and nonmyristoylated matrix proteins. We established efficient one-step metal affinity purification that enabled to obtain pure myristoylated matrix proteins suitable for structural and functional studies.

Introduction

The major structural component of all retroviruses is polyprotein precursor Gag. Depending on the genus of the retrovirus, Gag molecules assemble into immature viral particles by two distinct ways. Whereas betaretroviruses (e.g., mouse mammary tumor virus, MMTV[1]; Mason-Pfizer monkey virus, M-PMV) assemble in the cytoplasm prior to the budding from the host cell, most other genera like gammaretroviruses (e.g., murine leukemia virus, MLV) or lentiviruses (e.g., human immunodeficiency virus 1, HIV-1) assemble at the plasma membrane simultaneously with the budding. During the budding or shortly after the release of immature particles, the viral encoded protease cleaves the Gag polyprotein into individual structural domains. The key role in the choice of the assembly and budding site plays the N-terminal domain of Gag, the matrix protein (MA). It is the interaction of the MA with phospholipid membranes and other cellular components (e.g., Tctex-1, a light chain of the molecular motor dynein [1]) that drives the trafficking of assembled or single Gag molecules.

On its interaction surface, MA has positively charged amino acids that mediate the interaction with the negatively charged heads of the phospholipids in the membranes [2–4]. Furthermore, the MAs (of at least some retroviruses) are also capable to interact with the hydrophobic tails of the phospholipids [5–7]. Phosphatidylinositol 4,5-bisphosphate, PI(4,5)P_2, is considered to be the major factor that directs Gag specifically to the plasma membrane [8,9]. In addition to the charged amino acids, the MAs of most retroviruses are N-terminally myristoylated. Structural studies of the HIV-1 MA indicated that the myristoyl is sequestered in the protein prior to the interaction with the plasma membrane [6,10]. During the interaction, the myristoyl is exposed and inserted into the membrane, thus strengthening the binding of Gag to the membrane. This process, called a myristoyl switch, is well known from various membrane-binding proteins, e.g., ADP ribosylation factors [11] or recoverin [12]. Although the precise mechanism of the interaction with the membranes and the exact role of the myristoyl are not clear, it is certain that the myristoylation is crucial for the transport of Gag (assembled into viral particles or not) to the plasma membrane. The prevention of the myristoylation by the mutation of the N-terminal glycine to a valin in the M-PMV Gag interrupted the transport of assembled immature particles to the plasma membrane, retaining them in the perinuclear region of the cells [13]. In HIV-1 and the Moloney murine leukemia virus,

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[1] *Abbreviations used:* HSQC, heteronuclear single quantum correlation; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; MLV, murine leukemia virus; MMTV, mouse mammary tumor virus; M-PMV, Mason-Pfizer monkey virus; MS, mass spectrometry; myr(+), myristoylated; myr(−), nonmyristoylated; NMT, N-myristoyltransferase.

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the mutation or deletion of the N-terminal glycine led to the accumulation of unassembled Gag in the cytoplasm of the host cell [14,15].

To further investigate the general mechanism of the interaction of the MA with phospholipid membranes and to study the role of myristoyl in this process, it is necessary to use myristoylated MAs. However, large amounts of the myristoylated protein samples (ca. 10–20 mg) are often required for structural studies. Up to now, the only myristoylated MAs studied *in vitro* have been those of HIV-1 [10], HIV-2 [16], and M-PMV [5]. Protein N-myristoylation is catalyzed by N-myristoyltransferase (NMT), an enzyme that transfers the myristoyl from myristoyl-CoA to the N-terminal glycine of a suitable protein substrate. This protein modification is common in eukaryotes but is absent in prokaryotes. Although *Escherichia coli* does not have any innate myristoylation system, it is possible to introduce this ability using a recombinant NMT [17]. This approach makes *E. coli* a suitable and inexpensive source of myristoylated proteins. However, due to variable efficiency of the recombinant myristoylation, the production of myristoylated proteins in *E. coli* often results in a mixture of both myristoylated and nonmyristoylated proteins. This mixture is usually separated by hydrophobic interaction chromatography, but finding a suitable column and appropriate conditions can be difficult for some proteins.

Here we present the production and one-step separation of the myristoylated and nonmyristoylated forms of the MLV and MMTV matrix proteins. We achieved the concentration and purity of the myristoylated matrix proteins suitable for structural studies by NMR spectroscopy and X-ray crystallography.

Materials and methods

Construction of expression vectors

The DNA fragment encoding first 99 N-terminal amino acids of the MLV MA was obtained by PCR amplification of human prostate tumor cell cDNA (Rv1 cell line) using 5′-AAA AAA CAT ATG GGA CAG ACC GTA ACT-3′ and 5′-AAA AAA CTC GAG CGG TTT GAC CCA CGG A-3′ as a forward and reverse primer, respectively. The fragment encoding first 104 N-terminal amino acids of the MMTV MA was obtained by PCR using proviral clone HYB-MMTV [18] as a template and using 5′-GCC ATT GCA TAT GGG GGT CTC GGG CTC A-3′ and 5′-GGT AAC CTC GAG GGC TTC TGC GGA TAG CAA AAC CAA GTC-3′ as a forward and reverse primer, respectively. The fragments were digested with *Nde*I and *Xho*I (New England Biolabs) and subcloned to pET-22b plasmid (Novagen), thus adding a histidine-tag to the protein C-terminus. The resulting vectors were amplified in *E. coli* DH5α (Invitrogen) cells and verified by sequencing.

Expression of myristoylated proteins

Competent *E. coli* BL21 (DE3) cells (Invitrogen) containing pETyNMT vector for expression of N-myristoyltransferase [19] were transformed with the plasmid encoding either the MLV or MMTV MA with the histidine-tag. The transformed cells were used to inoculate 20 ml of LB medium (Sigma) and the suspension was then incubated overnight at 37 °C. Fresh LB medium (1 l) was inoculated with the overnight suspension, grown to OD_{590} 0.4–0.5 and then supplemented with sodium myristate (0.06 mM). After 30 min of incubation, expression was induced by 0.4 mM IPTG for 4 h. All procedures were carried out at 37 °C. The cells were harvested by centrifugation at 10,000*g* for 10 min.

Cell lysis

Cell pellets from 1 l of LB medium were resuspended in 50 ml of lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, a tablet of complete protease inhibitor cocktail (Roche), 10 mg of lysozyme (Sigma–Aldrich), pH 8) and incubated at room temperature for 30 min. Sodium deoxycholate was added to the final concentration of 0.2% (w/v) and the suspension was incubated for additional 30 min. The suspension was then sonicated and the insoluble fraction was removed by centrifugation at 20,000*g* for 20 min.

Immobilized metal affinity chromatography

The supernatant after the cell lysis was loaded onto an immobilized metal affinity chromatography column (HiTrap IMAC FF 5 ml, GE Healthcare) charged with Ni^{2+} and equilibrated with an IMAC buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) containing 10 mM imidazole. The column was washed with 25 ml of the IMAC buffer containing 58 mM imidazole and the MAs were eluted with increased concentration of imidazole, specific for individual proteins as described in Results, in the IMAC buffer. The concentration was optimized for each protein to give the best separation of the myristoylated, myr(+), and nonmyristoylated, myr(−), forms of the MA. The fractions were analyzed by SDS–PAGE and mass spectrometry.

Mass spectrometry

The samples were dialyzed against MS buffer (50 mM Na_2HPO_4, 50 mM NaCl, pH 8) and 1 μl of the dialyzed sample was mixed with 1 μl of matrix solution (10 mg/ml of sinapinic acid, 50% acetonitrile, 0.1% TFA). The spectra were obtained using ultrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics) set in a linear positive mode within the range of 4500–32,000 Da. The spectrometer was calibrated with Protein Calibration Standard I (Bruker Daltonics). The spectra were analyzed using mMass [20]. The approximate ratio of myr(−) MA and myr(+) MA in the mixture was derived from the intensities of the peaks in the spectra.

NMR spectroscopy

The protein samples for NMR spectroscopy were prepared as mentioned above with the following exception. Instead of LB medium, the cells were grown in M9 minimal medium [21] supplemented with [U–^{15}N]NH_4Cl as a sole source of nitrogen. The appropriate fractions after the IMAC separation were dialyzed against the NMR buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 6) and concentrated to 12 mg/ml. The NMR data were collected at 298 K on a Bruker Avance III 600 spectrometer equipped with a cryogenic triple-resonance probe. The ^{1}H–^{15}N HSQC spectra were measured with 2048 complex points and spectral width 9615.385 Hz for ^{1}H, and 128 complex points and spectral width 1581.259 Hz for ^{15}N. The data were processed with Topspin (Bruker BioSpin) and analyzed with CcpNmr Analysis [22].

Results

Expression of myristoylated MMTV and MLV matrix proteins

The MLV and MMTV MAs, fused with the C-terminal histidine-tag, were produced in *E. coli* coexpressing a yeast N-myristoyltransferase. The cells were grown in LB medium supplemented with sodium myristate. Whereas the MMTV MA was produced at relatively high amount (lane 3 in Fig. 1B), the production of the MLV MA was significantly lower (lane 3 in Fig. 1A). Both proteins