Biochimica et Biophysica Acta 1790 (2009) 1345–1352

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Requirement of the SH4 and tyrosine-kinase domains but not the kinase activity of Lyn for its biosynthetic targeting to caveolin-positive Golgi membranes

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ARTICLE INFO

Article history:
Received 24 March 2009
Received in revised form 18 June 2009
Accepted 13 July 2009
Available online 18 July 2009

Keywords:
Golgi caveolin
Kinase domain
Lipid modification
Lyn
Src-family tyrosine kinase
Targeting

ABSTRACT

Background: The Src-family non-receptor-type tyrosine kinase Lyn, which is often associated with chemotherapeutic resistance in cancer, localizes not only to the plasma membrane but also Golgi membranes. Recently, we showed that Lyn, which is synthesized in the cytosol, is transported from the Golgi to the plasma membrane along the secretory pathway. However, it is still unclear how Golgi targeting of newly synthesized Lyn is regulated.
Methods: Subcellular localization of Lyn and its mutants was determined by confocal microscopy.
Results: We show that the kinase domain, but not the SH3 and SH2 domains, of Lyn is required for the targeting of Lyn to the Golgi, whereas the N-terminal lipids of the Lyn SH4 domain are not sufficient for its Golgi targeting. Although intact Lyn, which colocalizes with caveolin-positive Golgi membranes, can traffic toward the plasma membrane, kinase domain-deleted Lyn is immobilized on caveolin-negative Golgi membranes.
General significance: Besides the SH4 domain, the Lyn kinase domain is important for targeting of newly synthesized Lyn to the Golgi, especially caveolin-positive transport membranes. Our results provide a novel role of the Lyn catalytic domain in the Golgi targeting of newly synthesized Lyn in a manner independent of its kinase activity.

1. Introduction

Src-family kinases, a family of non-receptor-type tyrosine kinases, consist of proto-oncogene products and structurally related proteins, and include at least eight highly homologous proteins: c-Src, Lyn, Fyn, c-Yes, c-Fgr, Hck, Lck, and Blk [1]. Src-family kinases are known to play crucial roles in regulating cell proliferation and differentiation [1]. Overexpression and enhanced activity of Src-family kinases found in a large number of human malignancies have been linked to the development of cancer and progression to metastases [2,3], and there has recently been a renewed interest in Src-family kinases as molecular targets for cancer therapy [4].

Src-family kinases, classified as cytosolic enzymes, are localized at the cytoplasmic face of the plasma membrane through posttranslational lipid modification [5], but an appreciable fraction is found at intracellular compartments, such as lysosomes, the Golgi apparatus and the nucleus [6–10]. Recent evidence suggests a correlation of Src localization with its function in cancer cells. For example, the activity of nuclear c-Src is associated with response to hormone therapy and patient survival in human ER-positive breast cancers [11]. We recently showed that the Src localization to late endosomes/lysosomes plays an important role for cancer in alteration of lysosome distribution [12]. Our studies also showed that Lyn, a member of Src-family kinases, is transported to the plasma membrane via the Golgi pool of caveolin along the secretory pathway [8]. We further showed that Lyn phosphorylates annexin II on the Golgi apparatus, leading to the translocation of annexin II from the Golgi to the endoplasmic reticulum [13]. Lyn is emerging as an oncogenic kinase in development of human leukemia and chemotherapy resistance [14–16], and it is suggested that anomalous subcellular localization of Lyn is associated with tumor pathogenesis [17]. Although Golgi localization of Lyn is required for its localization to the plasma membrane, the mechanism of Golgi targeting of Lyn has not been fully elucidated.

In this study, we show that besides the N-terminal lipids, the kinase domain but not the kinase activity of Lyn is required for the targeting of newly synthesized Lyn to the Golgi, especially to the Golgi pool of caveolin. Lack of the Lyn kinase domain induces immobilization of Lyn on caveolin-negative Golgi membranes and inhibits the trafficking toward the plasma membrane. Therefore, the kinase domain of Lyn plays an indispensable role in its targeting to caveolin-positive Golgi membranes after biosynthesis and subsequent trafficking toward the plasma membrane.

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doi:10.1016/j.bbagen.2009.07.009

2. Materials and methods

2.1. Plasmids

cDNA encoding human wild-type p56 Lyn ([19]; provided by T. Yamamoto) and its mutants were subcloned into the pcDNA4/TO vector (Invitrogen). The Trp→Ala mutation at position 99 [Lyn (W99A)-HA, Lyn118(W99A)-GFP and Lyn244(W99A)-GFP] was generated by site-directed mutagenesis using the primers as described previously [20]. The Arg→Ala mutation at position 156 [Lyn(R156A)-HA] was generated by site-directed mutagenesis using the sense primer 5′-CTGGAGCTTTCCTTATTGCAGAAAGTGAAACGTTAAAAG-GAAGCTTCTC-3′ and the antisense primer 5′-GAGAAGCTTCCTTT-TAACGTTTCACTTTCTGCAATAAGGAAAGCTCCAG-3′. LynΔSH3ΔSH2-HA and Lyn(C3S)ΔSH3ΔSH2-HA were generated from Lyn-HA and Lyn(C3S)-HA by deleting the sequence of the SH3 and SH2 domains. LynΔK-HA and LynΔK-GFP were constructed as described previously [8,9]. Lyn(SH4-Unique)-GFP was constructed as described previously [18]. Lyn118-GFP and Lyn244-GFP were constructed by fusion with GFP taken from pEGFP-C1 (CLONTECH Laboratories, Inc.) at the COOH terminus of the sequence of Lyn mutant (encoding 118 or 244 amino acid residues).

2.2. Antibodies

The following antibodies were used: mouse monoclonal anti-HA (F-7; Santa Cruz Biotechnology) and anti-GM130 (BD Transduction Laboratories) antibodies and rabbit polyclonal anti-caveolin (BD Transduction Laboratories) antibody. FITC-conjugated $F(ab')_2$ fragment of anti-mouse IgG or anti-rabbit IgG antibody, and TRITC-conjugated anti-mouse IgG or anti-rabbit IgG antibody were obtained from Biosource International and Sigma-Aldrich.

2.3. Cells and transient transfection

COS-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Cells were transiently transfected with pcDNA4/TO encoding Lyn mutants using TransIT Transfection reagent (Mirus) or linear polyethylenimine (25 kDa), according to the manufacturer's instructions, as reported previously [8,9,20,21]. For inhibition of the secretory pathway, cells were cultured at 19 °C for 2 or 3 h.

2.4. Immunofluorescence

Immunofluorescent staining was performed as described [8,9,20,21]. In brief, COS-1 cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min at room temperature, and permeabilized in PBS containing 0.1% saponin and 3% bovine serum albumin. Cells were subsequently reacted with an appropriate primary antibody for 1 h, washed with PBS containing 0.1% saponin, stained with FITC- or TRITC-conjugated secondary antibody for 1 h, and mounted with Prolong Antifade™ reagent (Molecular Probes). For staining of nuclei, cells were treated with 200 μg/ml RNase A and 100 μg/ml propidium iodide (PI) for 30 min. Confocal and Nomarski differential-interference-contrast images were obtained using a Fluoview FV500 laser scanning microscope with a 40×1.00 NA oil objective (Olympus, Tokyo). Care was taken to ensure that detection sensitivity was kept constant and that there was no breed-through from the fluorescein signal into the red channel. One-planar (xy) section slice images with 0.6-μm thickness were shown. Orthogonal sections viewing axial directions (xz and yz) were created when all Z-series sections at 0.3–0.5 μm intervals were merged. Composite figures were prepared using Photoshop 5.0 and Illustrator 9.0 software (Adobe).

3. Results

3.1. Requirement of the kinase domain, but not the SH3 and SH2 domains, for the Golgi targeting of Lyn

We reported that newly synthesized Lyn is transported through the Golgi region to the plasma membrane along the secretory pathway [8]. The N-terminal lipid modifications in the SH4 domain of Lyn are indispensable for membrane association [10]. Since we also showed that palmitoylation at the Lyn SH4 domain plays a role in Golgi localization [9,18,22], we examined whether lipid modification at the SH4 domain of Lyn determines its Golgi targeting. We therefore created Lyn(SH4-Unique)-GFP from human Lyn and examined its localization. Intriguingly, Lyn(SH4-Unique)-GFP was localized to the plasma membrane, but not the Golgi, whereas Lyn-GFP was localized to the Golgi region (Fig. 1A) and particularly colocalized with Golgi caveolin (data not shown). To examine whether Lyn(SH4-Unique)-GFP rapidly

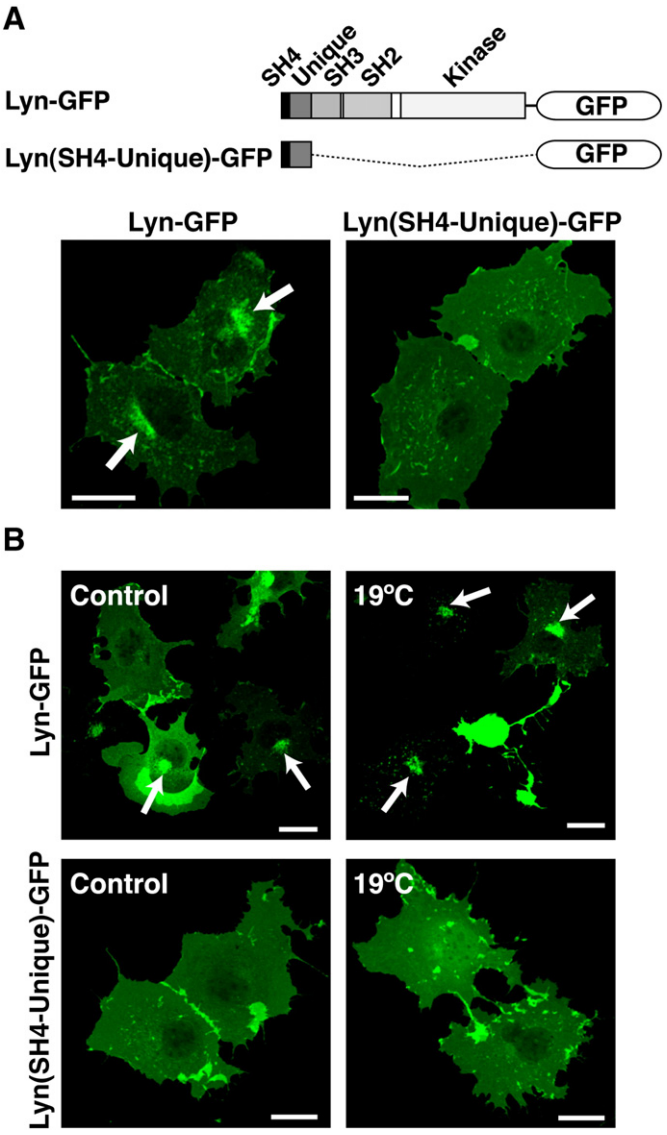

Fig. 1. Role of the N-terminal domain of Lyn in its Golgi targeting. (A) Schematic representations of Lyn-GFP and Lyn(SH4-Unique)-GFP are shown with the Src homology (SH) domains, the unique region, the kinase domain, and GFP. COS-1 cells transfected with each Lyn mutant were cultured for 18 h, and GFP fluorescence was visualized. (B) COS-1 cells transfected with Lyn-GFP or Lyn(SH4-Unique)-GFP were cultured for 18 h at 37 °C (Control) or 19 °C during the last 3 h, and GFP fluorescence was visualized. Arrows indicate Golgi localization of Lyn mutants. Scale bars, 20 μm.