Phosphorylation of the Vesicle Docking Protein p115 Regulates Its Association with the Golgi Membrane*

Miwa Sohda, Yoshiho Misumi, Akiko Yano, Noboru Takamiz, and Yukio Ikehara§

From the Department of Biochemistry and the Radioisotope Laboratory, Fukuoka University School of Medicine, Nanakuma, Jonan-ku, Fukuoka 814-80, Japan

The vesicle docking protein p115 was found to be phosphorylated in a cell cycle-specific manner; it was found phosphorylated in interphase but not in mitotic cells. During interphase, however, two forms of p115 were detected in the cells; the phosphorylated form was found exclusively in cytosol, whereas the unphosphorylated form was associated with membranes, mostly of the Golgi complex. The latter form was released from the membranes upon phosphorylation. Mutational analysis revealed that the phosphorylation site of p115 was the Ser942 residue in the C-terminal acidic domain. A mutant with a single substitution of Ser942 → Ala markedly increased its association with the Golgi membrane. Another mutant with Ser942 → Asp was able to associate with the membrane, although at a decreased level, indicating that the dissociation of p115 from the membrane is not simply due to the negative charge of phosphorylated Ser942. Taken together, these results suggest that the phosphorylation of Ser942 at the C-terminal acidic domain regulates the interaction of p115 with the Golgi membrane, possibly taking part in the regulatory mechanism of vesicular transport.

Vesicular transport of proteins is carried out by the formation of coated vesicles from a donor compartment, followed by their uncoating and subsequent docking and fusion of the vesicles with a target compartment membrane, in which a number of soluble and membrane proteins are involved (1). The docking of vesicles to target membranes is accomplished through the specific interaction between membrane proteins named v- and t-SNAREs (vesicle and target SNAP receptors) (1, 2). This is followed by binding of SNAPs (soluble N-ethylmaleimide-sensitive factor attachment proteins) and N-ethylmaleimide-sensitive factor, which lead to membrane fusion. Vesicle docking is also controlled by interactions of SNAREs with other proteins including Rab proteins (3).

p115, a peripheral membrane protein localized to the Golgi apparatus and also present in the cytoplasm, was first identified as a component required for intra-Golgi transport (4) and found to be identical to the transcytosis-associated protein TAP (5). Structural analysis also indicates that it is a homolog to Uso1p, a yeast protein required for transport from the endoplasmic reticulum to the Golgi (6). p115/TAP exits as a parallel homodimer with two globular heads followed by a rod-like domain containing a C-terminal acidic tail (5, 7). p115/TAP and Uso1p have been shown to function in a docking step prior to membrane fusion (8). Recently, Warren and his colleagues (9) demonstrated that p115 binds to Golgi membranes with high affinity during interphase but not during mitosis. In addition, GM130, a peripheral protein tightly associated with the cis-Golgi membrane (10), was identified as the binding site for p115 on the Golgi membrane (11). GM130 was modified by phosphorylation only during mitosis, resulting in no binding of GM130 to p115.

The mechanism of membrane binding inhibition by p115 during mitosis would provide a molecular explanation for the blocking of vesicular transport and for the vesicle-mediated fragmentation of the Golgi apparatus (12). During the interphase of cell cycle, however, p115 must recycle between membranes and cytosol to maintain vesicular transport. Because GM130 is stably associated with membranes and able to bind to p115 during interphase (9, 11), the recycling of p115 should be regulated by its own modification or by other factors. In the present study, we demonstrate that the membrane interaction of p115 is indeed regulated by phosphorylation, i.e. when not phosphorylated, p115 associates with the Golgi membrane but dissociates from the membrane upon its phosphorylation.

EXPERIMENTAL PROCEDURES

Preparation of Human p115 cDNA and Anti-p115 Antibodies—The full-length cDNA for human p115 was isolated from a liver cDNA library using a rat p115 cDNA fragment as a probe (5, 13). The cDNA (3.9 kilobase pairs) was found to encode a protein of 962 amino acids (108 kDa) that shows 95.3 and 91.9% identity with p115 of bovine and rat, respectively (5, 7). Two chimeric proteins of p115 fragments (amino acid numbers 644–730 or 744–855) fused to the C terminus of glutathione S-transferase were constructed in the expression vector pGEX3X (14). The recombinant proteins were expressed in and purified from bacteria and injected into rabbits to raise anti-p115 antibodies as described previously (13).

Construction and Transfection of Expression Plasmids—A cDNA covering the entire coding region of human p115 was inserted into the EcoRI site of pSG5 expression vector and designated pSG5/p115. The FLAG7 tag sequence encoding the amino acid sequence MDYKDDDDK was ligated in-frame into the BamHI site of pSG5/p115 that had been introduced at 11 bases upstream from the N-terminal ATG codon. Constructs of truncated p115 were generated by site-directed mutagenesis; the termination codon TAG was introduced into the Lys928 codon (AAG) for truncation of the C-terminal acidic tail domain (designated ΔAD) and also into the Glu947 codon (CAG) for truncation of both the rod-like coiled-coil domain and the acidic domain (designated ΔRAD). A construct lacking the N-terminal head domain was generated by in-frame ligation of the BgIII-XhoI cDNA fragment of pSG5/p115 downstream of the FLAG sequence (designated ΔHD). Mutants with the following substitutions were also prepared by site-directed mutagenesis of pSG5/p115: Ser942 (TCT) → Ala (GCT), Ser952 (AGT) → Ala (GCT), and Ser942 → Ala/Ser952 → Ala. All the constructs prepared by site-directed mutagenesis were verified by sequencing. Each plasmid (20...
Phosphorylation and Membrane Interaction of p115

µg) was transfected into COS-1 cells as described (13).

Metabolic Labeling, Immunoprecipitation, and SDS-Polyacrylamide Gel Electrophoresis—HeLa cells (4 × 10⁶ cells/dish) or transfected COS-1 cells (5 × 10⁶ cells/dish) were labeled at 37 °C for 5 h with [35S]methionine (4 MBq/dish) or with [³²P]orthophosphate (20 MBq/dish). When indicated, interphasic and mitotic cells that had been prepared according to Suprynowicz and Gerace (15) were also labeled as above. Cell lysates were prepared and subjected to immunoprecipitation with polyclonal anti-p115, anti-human GM130, or monoclonal anti-FLAG M2 antibodies (Eastman-Kodak, New Haven, CT). The immunoprecipitates were analyzed by SDS-PAGE¹ (7% gels) and fluorography (16).

Cell Fractionation and Immunoblotting—A postnuclear fraction was prepared from HeLa cells or transfected COS-1 cells and centrifuged at 105,000 × g for 1 h into membrane and cytosol fractions as described previously (13). A Golgi-enriched fraction was prepared by flotation of the postnuclear fraction in a sucrose gradient as described by Balch et al. (17). The samples, when indicated, were incubated at 37 °C for 30 min in the presence or the absence of calf intestinal alkaline phosphatase (50 units/ml) in 50 mM Tris-HCl (pH 9.0). Each sample was analyzed by SDS-PAGE (7% gels) or by isoelectric focusing (18, 19) followed by immunoblotting with the indicated antibodies (at dilution 1:1000 of each antibody). The immunoreactive proteins were visualized using the ECL kit (20). In some experiments the immunoblots were scanned with a G鲜明5500 scanner (Epson, Inc., Tokyo) and analyzed by Adobe Photoshop (Adobe Photosystems, Columbia, MD) and NIH Image software (20).

In Vitro Phosphorylation—A cytosol fraction was prepared from the postnuclear fraction of HeLa S3 cells by centrifugation at 105,000 × g for 1 h. An aliquot of the cytosol (100 µl of 5 mg protein/ml) was incubated with Sepharose CL-4B beads coupled with anti-p115 IgG at 4 °C overnight with gentle shaking. After spinning the beads down, the supernatant was adjusted to contain 25 mM Hepes-KOH (pH 7.2), 60 mM potassium acetate, 2.5 mM magnesium acetate, and a mixture of protease inhibitors (19) and used as the p115-depleted cytosol. The Golgi membrane (60 µg) was incubated at 37 °C for 10 min in 200 µl of a reaction mixture containing 25 mM Hepes (pH 7.2), 60 mM potassium acetate, 2.5 mM magnesium acetate, 400 µg of the control or p115-depleted cytosol, 50 µM Mg-ATP, and [γ-³²P]ATP (3.7 MBq). At the end of incubation, an equal volume of 2 concentrated lysis buffer (16) was added into the reaction mixture, followed by immunoprecipitation and SDS-PAGE as described above.

RESULTS

Phosphorylation of p115—Total cell lysates prepared from HeLa cells that had been labeled with [35S]methionine or [³²P]P, were immunoprecipitated with anti-p115 antibodies. The immunoprecipitate contained a single ³²P-labeled protein with the expected molecular size (Fig. 2B, lanes 2–5). When the transfected cells were incubated with [³²P]P, only the mutant containing the C-terminal acidic domain, but not the other mutants, was heavily labeled (Fig. 2B, lanes 7–10). The result indicates that the phosphorylation site(s) of p115 is contained in the C-terminal acidic domain.

The C-terminal acidic domain of human p115 contains no Tyr and Thr residues but contains two Ser residues at positions 942 and 952 (Fig. 2C). The possible phosphorylation of the Ser residues in the domain was examined by substitution of Ser → Ala in each position. The band labeled with [³²P]P was dramatically reduced in intensity in the mutant containing Ser⁹⁴² → Ala alone and was undetectable in the double mutant (Fig. 2D, lanes 2 and 4). In contrast, a substitution Ser⁹⁴⁵ → Ala alone caused no significant effect on the phosphorylation of p115 (Fig. 2D, lane 3). The results indicate that the Ser⁹⁴² residue is the predominant site of phosphorylation. In fact, the Ser⁹⁴⁵ but not the Ser⁹⁵² is conserved in p115 from other species (Fig. 2C), which was also found to be phosphorylated as shown in Fig. 1B.

Phosphorylation of p115 Regulates Its Association with Membranes—When a postnuclear fraction prepared from [³²P]P-labeled cells was separated into membrane and cytosol fractions, more than 90% of the [³²P]P-labeled p115 was recovered in the cytosol fraction (Fig. 3A, lanes 1 and 2). Immunoblot analysis, however, showed that the membrane fraction contained approximately one-third of the total p115 (Fig. 3A, lanes 3 and 4). The membrane-associated and cytosolic forms were found to have a different pI when analyzed by isoelectric focusing, and the more acidic cytosolic form was converted to a form with the same mobility as the membrane form by alkaline phosphatase treatment (Fig. 3A, lanes 5–8). These results indicate that the cytosolic p115 is the phosphorylated form, whereas the membrane-associated protein is the unphosphorylated one.

We then examined whether p115 is released from the Golgi membrane upon its phosphorylation in vitro. When the Golgi membrane was incubated with [³²P]P, p115 was phospho-

¹ The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
rlylated equally in the presence or the absence of the p115-depleted cytosol (Fig. 3B, lanes 2 and 3), suggesting that phosphorylation of p115 requires no cytosolic factor. When the reaction mixture was separated by centrifugation into a supernatant and a membrane pellet, all the 32P-labeled protein was recovered in the supernatant (Fig. 3B, lanes 4 and 5), suggesting that the protein is released from the membrane upon its phosphorylation.

We further examined the effect of mutations of the phosphorylation site Ser942 on the membrane association of p115. The wild-type p115 and mutants with a substitution of Ser942 → Ala or Ser942 → Asp were transfected into COS-1 cells, from which postnuclear and Golgi-enriched fractions were prepared. Immunoblot analysis of p115 in the postnuclear fraction demonstrated that all the wild-type and mutant proteins were expressed at a similar level in the transfected cells (Fig. 3C, lane 1, 3, and 5). Recovery of the mutants in the Golgi membrane was increased approximately 180% for the mutant with
Phosphorylation and Membrane Interaction of p115

Ser^{942} \rightarrow \text{Ala}, \text{whereas the mutant with Ser}^{942} \rightarrow \text{Asp was decreased by 50\%, compared with that of the wild type (Fig. 3D). The value obtained for the wild-type protein may reflect a steady state of its membrane association, possibly regulated by its phosphorylation and dephosphorylation. Lack of phosphorylation of the mutant with a Ser^{942} \rightarrow \text{Ala substitution could prevent its dissociation from the membrane, resulting in a 2-fold increase of the association, which may reflect a saturated level on the membrane. The results also suggest that the functional role of the phosphorylated Ser^{942} is not completely compensated by the negatively charged residue Asp.}

DISCUSSION

In the present study we demonstrated that p115 is phosphorylated in interphase cells but not in mitotic cells. This is in contrast to the phosphorylation of GM130 that takes place only during mitosis (9, 11). What is the functional significance of such a differential phosphorylation response of the two proteins in vesicular transport? Available evidence indicates that the N-terminal 73-residue domain of GM130 binds p115, whereas the C-terminal half is involved in binding to the Golgi membrane (11). The binding of GM130 to the Golgi membrane is unlikely to be regulated, because mitotic phosphorylation both in vivo (9) and in vitro (11) does not release GM130 from the membrane. Mitotic phosphorylation of GM130 at the N-terminal domain, however, prevents its association with p115 that mediates docking of transport vesicles to the membrane. This may explain why vesicular transport is blocked during mitosis and the Golgi membranes are concurrently fragmented (12, 21). The phosphorylation of GM130 and its inhibitory effect on the binding to p115 are blocked by treatment with cyclin-dependent kinase inhibitors (11). In fact, the N-terminal domain of GM130 contains two putative cyclin-dependent kinase phosphorylation sites (11).

The presence of p115 both in the cytosol and Golgi membrane suggests that p115, involved in the docking of vesicles to the membrane, is released from the membrane into the cytosol. The recycling of p115 may be essential to maintain active vesicular transport during interphase. Because GM130 is not phosphorylated during interphase and stably binds to p115, the recycling of p115 occurring during interphase cannot be regulated by GM130 itself. The data presented here support the idea that the interphase recycling of p115 is regulated by its own phosphorylation. p115, which primarily exists as a phosphorylated form in the cytosol, associates with the Golgi membrane when it is dephosphorylated, whereas the protein dissociates from the membrane upon its phosphorylation. The p115-binding protein on the Golgi membrane may be GM130 as suggested by Nakamura et al. (11), although we have not directly identified it. Thus, it is likely that during interphase the dephosphorylation of GM130 is a prerequisite for the initial binding of p115 to the Golgi membrane, which is regulated by phosphorylation and dephosphorylation of p115 itself. In contrast, during mitosis the phosphorylation of GM130 prevents the binding of p115 to the membrane (11) even when p115 is dephosphorylated.

p115 was phosphorylated predominantly at the Ser^{942} residue in the C-terminal acidic domain. The amino acid sequence surrounding Ser^{942}, however, does not correspond to any of the known phosphorylation consensus motifs (22). The lack of a requirement for cytosol during phosphorylation suggests that p115 is phosphorylated by a membrane-associated protein kinase, which is clearly different from that involved in phosphorylation of GM130. Further studies are required for the characterization of kinases and phosphatases for p115 and GM130, which may shed light on the regulatory mechanism of vesicular transport.

Acknowledgments—We thank Dr. J. L. Millan (the Burnham Institute) for critical reading of the manuscript, Dr. Yuko Misumi (Saga Medical School) for help and useful suggestions, and C. Hashimoto for technical assistance.

REFERENCES

1. Rothman, J. E. (1994) Nature 372, 55–63
2. Rothman, J. E., and Wieland, F. T. (1996) Science 272, 227–234
3. Nueffer, C., and Balch, W. E. (1994) Annu. Rev. Biochem. 63, 849–900
4. Waters, M. G., Clary, D. O., and Rothman, J. E. (1992) J. Cell Biol. 118, 1015–1026
5. Barroso, M., Nelson, D. S., and Sztul, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 527–531
6. Nakajima, H., Hirata, A., Yonehara, T., Yoneda, K., and Yamashita, M. (1991) J. Cell Biol. 113, 245–260
7. Sapperstein, S. K., Walter, D. M., Grosvener, A. R., Heuser, J. E., and Waters, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 522–526
8. Sapperstein, S. K., Lupashin, V. V., Schmitt, H. D., and Waters, M. G. (1996) J. Cell Biol. 132, 755–767
9. Levine, T. P., Rabouille, C., Kieckbusch, R. H., and Warren, G. (1996) J. Biol. Chem. 271, 17304–17311
10. Nakamura, N., Rabouille, C., Watson, R., Nilson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995) J. Cell Biol. 131, 1715–1726
11. Nakamura, N., Lowe, M., Levine, T. P., Rabouille, C., and Warren, G. (1997) Cell 89, 445–455
12. Featherstone, C., Griffiths, G., and Warren, G. (1985) J. Cell Biol. 101, 2036–2046
13. Misumi, Y., Sobli, M., Yano, A., Fujiwara, T., and Ikehara, Y. (1997) J. Biol. Chem. 272, 23851–23858
14. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
15. Suprynowicz, F. A., and Gerace, L. (1986) J. Cell Biol. 103, 2073–2081
16. Misumi, Y., Oda, K., Fujiwara, T., Takami, N., Tashiro, K., and Ikehara, Y. (1991) J. Biol. Chem. 266, 16954–16959
17. Balch, W. E., Dunphy, W. G., BraeII, W. A., and Rothman, J. E. (1984) Cell 39, 405–416
18. Ames, G. F.-L., and Nikaido, K. (1976) Biochemistry 15, 616–623
19. Misumi, Y., Miki, K., Takami, N., Tashiro, K., and Ikehara, Y. (1991) J. Biol. Chem. 266, 16954–16959
20. Harata, T., Takami, N., Ohmura, M., Misumi, Y., and Ikehara, Y. (1997) Biochem. J. 325, 455–463
21. Lucoq, J. M., Pryde, J. G., Berger, E. G., and Warren, G. (1987) J. Cell Biol. 104, 865–874
22. Songyang, Z., Lu, K., Kwon, Y. T., Tsai, L.-H., Filhol, O., Cochet, C., Bricey, D. A., Soderling, R., Baltleson, C., Graves, D. J., DeMaggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, T., and Catley, L. C. (1996) Mol. Cell. Biol. 16, 6486–6493