# A critical period requiring Rho proteins for cell cycle progression uncovered by reversible protein transduction in endothelial cells

Katsuya Hirano[a,*], Mayumi Hirano[a], Junji Nishimura[a], Hideo Kanaide[a,b]

[a]*Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[b]*Kyushu University COE Program on Lifestyle-Related Diseases, Kyushu University, Fukuoka, Japan*

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**Abstract The time-specific requirement of Rho proteins for the S phase progression of vascular endothelial cells was determined by reversibly introducing inhibitor proteins with a cell-penetrating peptide. We found evidence of the reversibility of protein transduction. The removal of extracellular protein caused the transduced protein to decay in a manner sensitive to low temperatures. The time required for a 50% decay correlated with the protein size. The time-specific transduction of the inhibitor proteins uncovered a critical period requiring Rho proteins in the $G_1$–S transition phase. Reversible protein transduction may thus be a powerful tool to investigate the time-specific role of signaling proteins.**


*Keywords:* Cell-penetrating peptide; Endothelial cell; Cell cycle; Rho protein

## 1. Introduction

Rho proteins, RhoA, Rac1, and Cdc42, have been shown to promote cell cycle progression to the S phase [1]. However, it remains to be determined precisely when they are required for such S phase progression. It is difficult to examine such a time-specific role of Rho proteins by conventional transfection experiments using constitutive active or dominant negative mutants, because there is a significant time delay in both the increase and decrease in the level of protein expression. On the other hand, cell-penetrating peptides such as those found in human immunodeficiency viral Tat protein have been successfully utilized to rapidly introduce proteins into living cells with an intact plasma membrane, thus resulting in significant biological effects [2–5]. Furthermore, the native Tat protein was shown to exit the cells infected by human immunodeficiency virus and to enter the neighboring uninfected cells [5,6]. The intraperitoneal injection of β-galactosidase conjugated with a protein transduction domain (PTD) of Tat was shown to be delivered to all tissues in mice including brain tissue [7]. We observed that the enhancement of arterial contraction by introducing a dominant negative mutant of MYPT1, a regulatory subunit of smooth muscle myosin phosphatase, was reversible and reproducible [8]. These observations thus suggested that the intracellularly transduced cell-penetrating peptide could exit the cells, and therefore protein transduction could be reversible. The Tat PTD-mediated protein transduction is thus anticipated to make it possible to examine the time-specific role of Rho proteins in the cell cycle progression.

The protein transduction process was initially thought to be independent of receptor, transporter or endocytosis, and resistant to a low temperature and ATP-depleted conditions [3–5,9]. However, there is still controversy regarding the involvement of endocytosis [10–12], energy-dependency [10] and a specific "receptor" for entry [13,14]. Moreover, the mechanism for protein transduction could be different depending on the type of cell-penetrating peptides and target cells, as well as in situations where the peptides were free or conjugated with cargo [4,5,15]. However, no evidence regarding the reversibility of protein transduction has yet been obtained, and both the characteristics and kinetics of protein extrusion remain to be elucidated.

The present study thus investigated the reversibility of Tat PTD-mediated protein transduction in the bovine aortic endothelial cells (BAECs) and elucidated some characteristics of the protein extrusion process. Next, by introducing RhoA- and Rac1/Cdc42-inhibitory proteins in a time-specific manner, we, for the first time, determined the critical period of the cell cycle that requires RhoA and Rac1/Cdc42 for S phase progression.

## 2. Materials and methods

### 2.1. Construction, expression and purification of the recombinant protein

The vectors used to express PTD-tagged proteins with and without a hemagglutinin (HA) tag were pTAT, pTATHA, pQE30TAT and pQE30TATHA [8,16]. pQE30 (Qiagen, Hilden, Germany) was used to express the control $(His)_6$-tagged proteins without PTD. The cDNA encoding green fluorescence protein (GFP) was obtained from pEGFP-N1 (Clontech, Palo Alto, CA, USA). The cDNAs encoding the RhoA-binding domain (RB) of Rho-kinase (amino acid residues 941–1075 [17]) and the Rac1, Cdc42-binding domain (PBD) of

* Corresponding author. Fax: +81-92-642-5552.
*E-mail address:* khirano@molcar.med.kyushu-u.ac.jp (K. Hirano).

*Abbreviations:* BAECs, bovine aortic endothelial cells; GFP, green fluorescence protein; HA, hemagglutinin; PBD, Rac1/Cdc42-binding domain of p21-activated protein kinase; PTD, protein transduction domain; RB, RhoA-binding domain of Rho-kinase

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p21-activated protein kinase 1 (amino acid residues 67–150 [18]) were obtained from the human aorta cDNA library (Clontech). The cDNA encoding IgG-binding domain of protein A (amino acid residues 23–270 [19]) was obtained from pMALU5 [20]. pMALU5 was kindly donated by Dr. M. Aizawa (Tokyo Institute of Technology, Yokohama, Japan). All inserts were obtained by PCR amplification and they were determined to contain no unintended mutation. The recombinant proteins were expressed and purified with the $Ni^{2+}$-loaded Hi-Trap chelating column on Akta Prime (Pharmacia, Tokyo, Japan) as previously described [8]. The protein concentration was determined with a Coomassie protein assay kit (Pierce, Rockford, IL, USA).

### 2.2. Cell culture and analysis of cell cycle progression

The BAECs were cultured as described [21]. The cells were plated at a confluent density of 25% and the progression of the S phase of the cell cycle was evaluated by propidium iodide staining as previously described [22].

### 2.3. Protocol for protein transduction and extrusion

The cells were mechanically harvested from the culture dishes and collected in PBS. The cells (at approximately $5 \times 10^6$ cells/ml) were incubated in PBS containing 1 μM of recombinant proteins for 15 min at 37 °C (transduction step). The cells were then collected by brief centrifugation and thoroughly washed in ice-cold PBS. The cells were resuspended in PBS at $5 \times 10^5$ cells/ml and then incubated at the indicated temperature for the indicated period of time (extrusion step). When the effect of metabolic inhibition was examined, the cells were incubated for 30 min at 37 °C with and without (control) 1 μM of rotenone and 10 mM of 2-deoxy-D-glucose [23] in the continuous presence of recombinant proteins after a 15-min transduction step. The cells were then subjected to the extrusion step with and without metabolic inhibition. To remove any possible Tat-tagged proteins attached to the extracellular surface [10], the cells were equilibrated for 5 min at 4 °C after the 15-min transduction step and were then treated with trypsin (bovine pancreas, Sigma) at 50:1 mass ratio of substrate to enzyme at 4 °C for 10 min. Under this condition, TATHA-RB was completely proteolyzed in vitro (data not shown). The trypsin digestion was then terminated by adding 10 μM 4-aminido-phenylmethanesulfonyl fluoride and by thoroughly washing in ice-cold PBS before proceeding to further analyses.

### 2.4. Immunoblot detection of recombinant proteins

The intracellularly transduced proteins were detected as described [8]. The protein extruded in the bathing buffer was recovered and concentrated by passing the buffer through $Ni^{2+}$-nitrilo acetate resin (Qiagen). The recombinant proteins were detected by anti-$(His)_6$ antibody as previously described [8]. The density of bands detected by anti-$(His)_6$ antibody was normalized by the density of tubulin or actin in the corresponding samples, and then the relative density at time 0 was assigned to be 100%.

### 2.5. Flow cytometric analysis of GFP fluorescence

At each time point of the extrusion step, an aliquot of the cell suspension ($1.6 \times 10^5$ cells) was directly subjected to flow cytometry, and GFP fluorescence was analyzed as previously described [24].

### 2.6. Statistics

All data were expressed as means ± S.D. The time required for a 50% decay was estimated by fitting the decay curve to exponential regression. The unpaired Student's *t*-test evaluated statistical significance. $P < 0.05$ was considered to be significant.

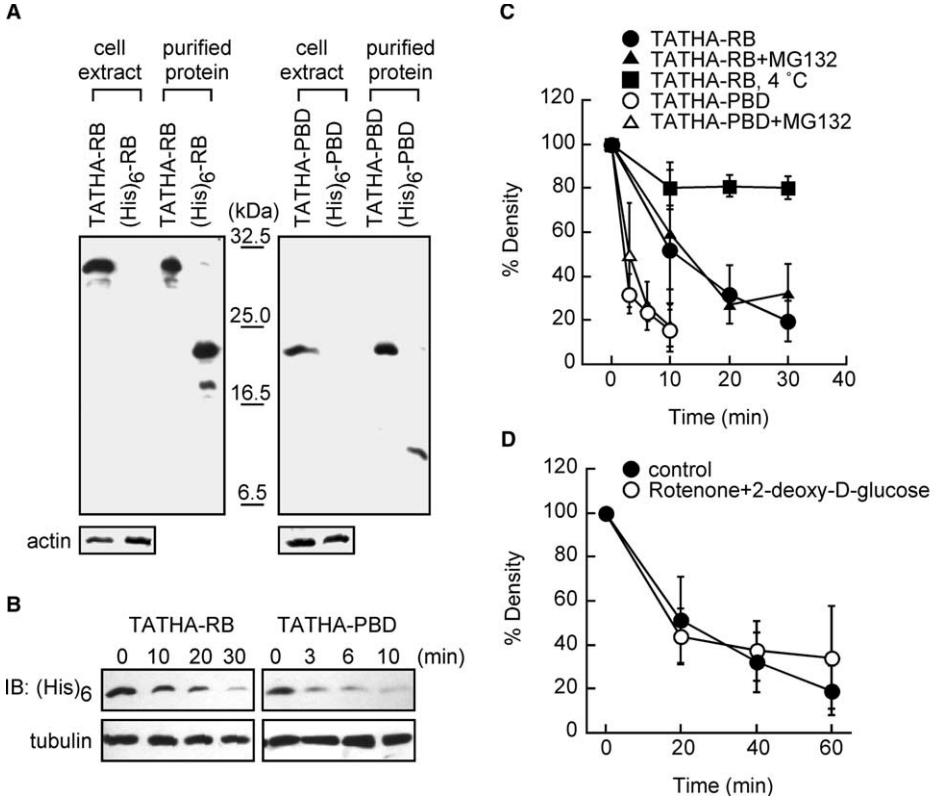


Fig. 1. Reversible protein transduction in BAECs. (A) Immunoblot detection of the recombinant proteins with anti-$(His)_6$ antibody in the extract of the cells exposed to 1 μM TATHA-RB (25.8 kDa), $(His)_6$-RB (19.0 kDa), TATHA-PBD (19.6 kDa) and $(His)_6$-PBD (12.8 kDa) for 15 min at 37 °C. The purified protein (100 ng) was loaded as a positive control. Actin was detected with naphthol blue black staining to validate equal loading of the cell extracts. (B) Time course of decay of TATHA-RB and TATHA-PBD at 37 °C. The cells had been exposed to 1 μM recombinant proteins for 15 min at 37 °C. Tubulin was detected by a specific antibody to validate the equal loading of the cell extracts. (C) The effect of 10 μM cell-permeable proteasome inhibitor MG132 and a low temperature (4 °C) on the extrusion of TATHA-RB and TATHA-PBD. (D) The decay of TATHA-RB with and without (control) metabolic inhibition (1 μM rotenone + 10 mM 2-deoxy-D-glucose). The data are means ± S.D. ($n = 3$).

## 3. Results

### 3.1. Reversible protein transduction in BAECs

The intracellular transduction of RB and PBD was verified by immunoblot detection (Fig. 1A) as described [8,25,26]. After a 15 min incubation, RB and PBD were detected in the extracts only when they were conjugated with Tat PTD (Fig. 1A; TATHA-RB and TATHA-PBD). The subsequent removal of the recombinant proteins in the bathing buffer caused a time-dependent decrease in the amount of recombinant proteins detectable in the cell extract (Fig. 1B). The time required for a 50% decline of TATHA-RB (25.8 kDa) and TATHA-PBD (19.6 kDa) at 37 °C was estimated to be 12.9 and 3.9 min, respectively (Fig. 1C). On the other hand, a cell-permeable proteasome inhibitor MG132 (10 μM) had no effect on the decay of TATHA-RB and TATHA-PBD (Fig. 1C). Lowering the temperature during the extrusion step markedly inhibited the decay of TATHA-RB (Fig. 1C). However, the metabolic inhibition with 1 μM rotenone and 10 mM 2-deoxy-D-glucose [23] had no effect on the decay of TATHA-RB (Fig. 1D).

To rule out any possible involvement of Tag-tagged proteins attached to the extracellular surface in the observations shown in Fig. 1 [10], the cells were treated with trypsin after a 15-min transduction step (Fig. 2). Trypsin treatment reduced the amount of TATHA-RB in the cell extract to ~40% of that seen without trypsin treatment (Fig. 2A). However, the intracellularly transduced TATHA-RB declined after removal of the extracellular TATHA-RB with a similar time course to that seen without trypsin treatment, and the time required for a 50% decline was 13.1 min (Fig. 2B). Lowering the temperature during the extrusion step markedly inhibited the decay of TATHA-RB as observed without trypsin treatment (Fig. 1C). Corresponding to the decrease in intracellular TATHA-RB, TATHA-RB was detected in the bathing buffer (supernatant) after a 30 min incubation in PBS at 37 °C (Fig. 2C).

### 3.2. Decay of TATHA-GFP in BAECs

The extrusion of the transduced proteins was also examined with TATHA-GFP (Fig. 3). The cells were first exposed to 1 μM TATHA-GFP for 15 min at 37 °C and then extracellular TATHA-GFP was removed. The intensity at the peak of fluorescence distribution thereafter decreased in a time-dependent manner at 37 °C (Fig. 3A). The time required for a 50% decay of TATHA-GFP (37.3 kDa) was estimated to be 34.8 min at 37 °C (Fig. 3B). Lowering the temperature to 4 °C during the extrusion step markedly inhibited the decay of GFP

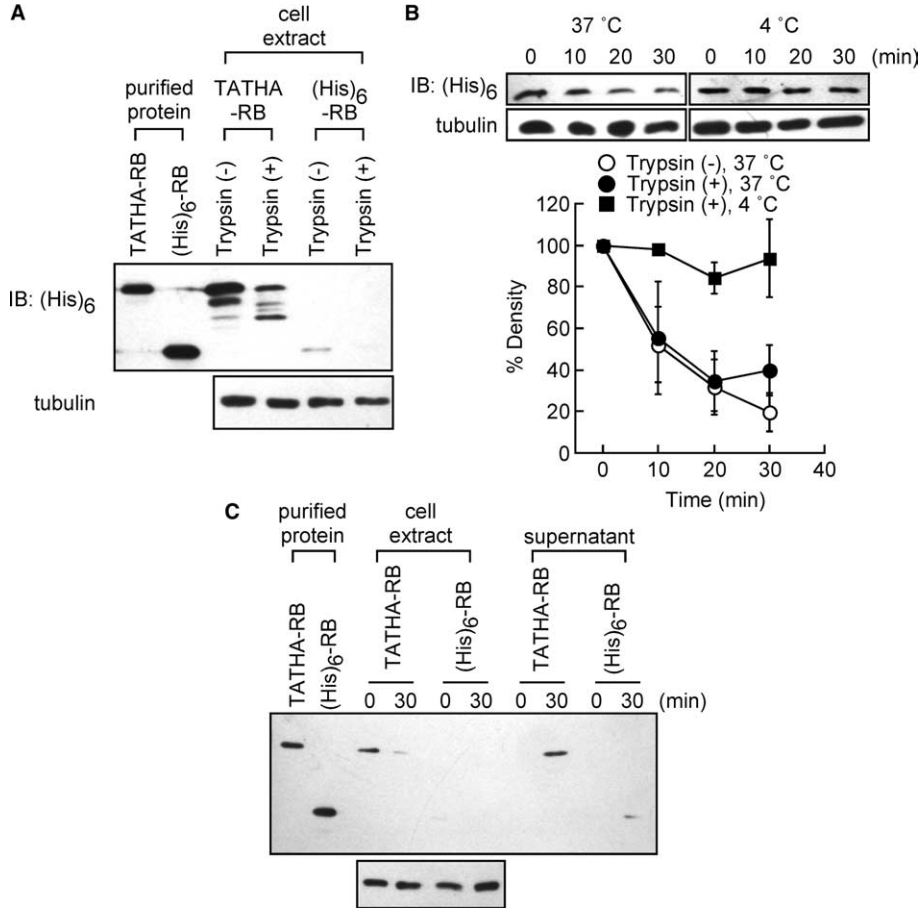


Fig. 2. Immunoblot analyses of protein transduction and extrusion after trypsin treatment. (A) Immunoblot detection of the recombinant proteins with anti-$(His)_6$ antibody in the extract of the cells exposed to 1 μM TATHA-RB and $(His)_6$-RB for 15 min at 37 °C. The cells were treated with trypsin at 50:1 mass ratio of substrate to trypsin at 4 °C for 10 min before extracting cellular proteins. The purified protein (100 ng) was loaded as a positive control. Tubulin was detected to validate equal loading of the cell extract. (B) Representative photos and summary of the decay of TATHA-RB at 37 and 4 °C after trypsin treatment. The data for the decay at 37 °C without trypsin treatment are from Fig. 1C. The data are means ± S.D. ($n = 3$). (C) Immunoblot detection of TATHA-RB and $(His)_6$-RB in the cell extract and the bathing buffer (supernatant) at 0 and 30 min after removal of the extracellular proteins following trypsin treatment. Tubulin was detected to validate equal loading of the cell extract.

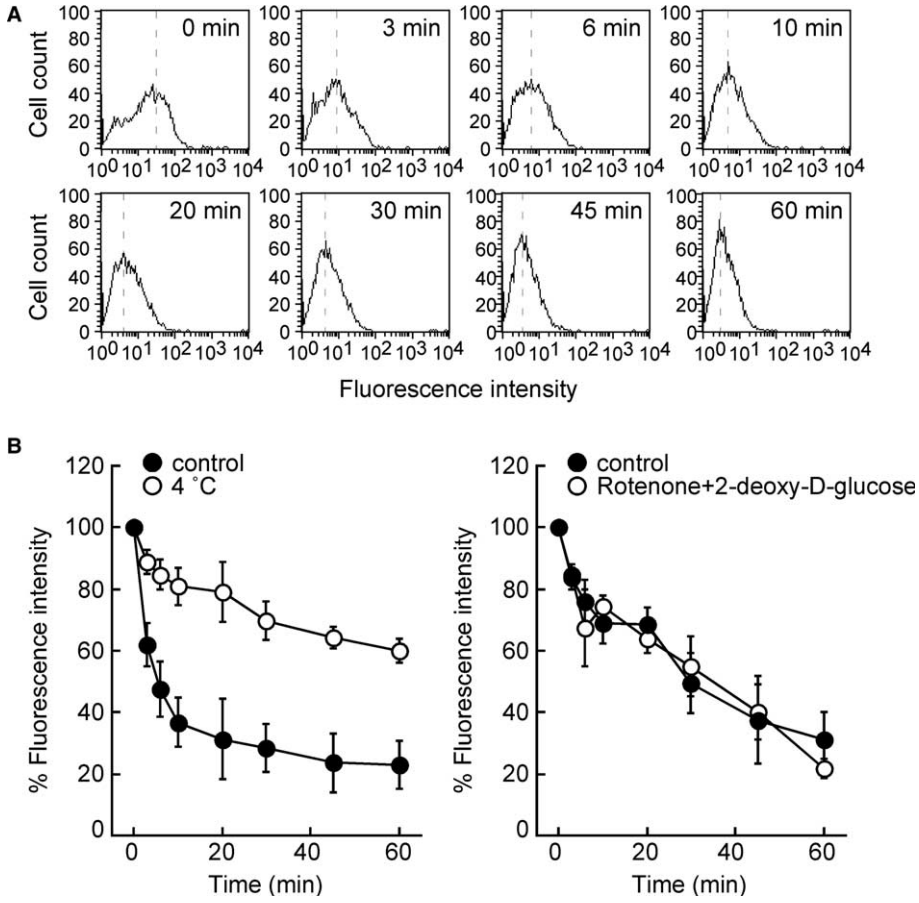


Fig. 3. Effect of a low temperature and metabolic inhibition on the decay of GFP in BAECs. (A) A representative histogram of a flow cytometric analysis of GFP fluorescence at the indicated time after removing extracellular TATHA-GFP at 37 °C. The cells had been exposed to 1 μM TATHA-GFP for 15 min at 37 °C. (B) The effect of a low temperature (4 °C) and metabolic inhibition (1 μM rotenone + 10 mM 2-deoxy-D-glucose) on the decay of GFP fluorescence intensity. The data are means ± S.D. ($n = 3$).

fluorescence, while the metabolic inhibition had no effect (Fig. 3B). These observations were consistent with those seen for TATHA-RB and TATHA-PBD (Figs. 1 and 2).

### 3.3. Correlation between the size of protein and the time required for a 50% decay

Plotting the time required for a 50% decay against the size of the protein revealed a positive correlation between them (Fig. 4). Within the molecular sizes examined (20–40 kDa), the following linear relationship was obtained:

$$\text{Time required for a 50\% decay (min)} = 1.7 \times \text{Molecular mass (kDa)} - 31.5$$

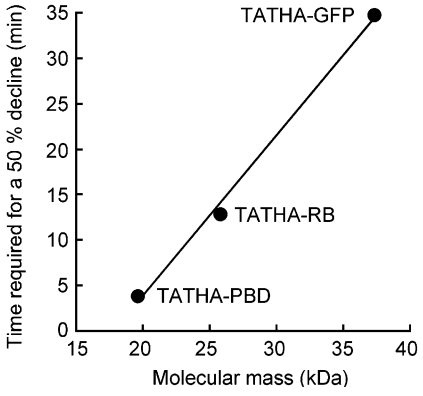


Fig. 4. Relationship between the molecular mass of the recombinant protein and the time required for a 50% decay in the amount of the introduced proteins. The time required for a 50% decay at 37 °C of TATHA-GFP (37.3 kDa), TATHA-RB (25.8 kDa) and TATHA-PBD (19.6 kDa) was plotted against the molecular mass. The relationship between the molecular mass (kDa) and time required for a 50% decay (min) was fitted to the linear regression ($R^2 = 0.99574$).

### 3.4. Time-specific inhibition of the cell cycle progression by the reversible transduction of RB and PBD

RB and PBS have been shown to serve as dominant negative mutants against endogenous RhoA and Rac1/Cdc42, respectively [27]. When BAECs were replated at a confluent density of 25%, the cells started to enter the S phase after 12 h and reached the peak of the S phase at 24 h (Fig. 5A). When RB and PBD conjugated with PTD either with or without HA were applied at time 0, the S phase progression was concentration-dependently inhibited (Fig. 5B). The removal of Tat PTD abolished the inhibitory effect ($(His)_6$-RB). The control proteins, TAT-GFP, TATHA-GFP and TATHA-protein A, had no effect, except that 100 nM TATHA-GFP showed a slight inhibition (Fig. 5B).

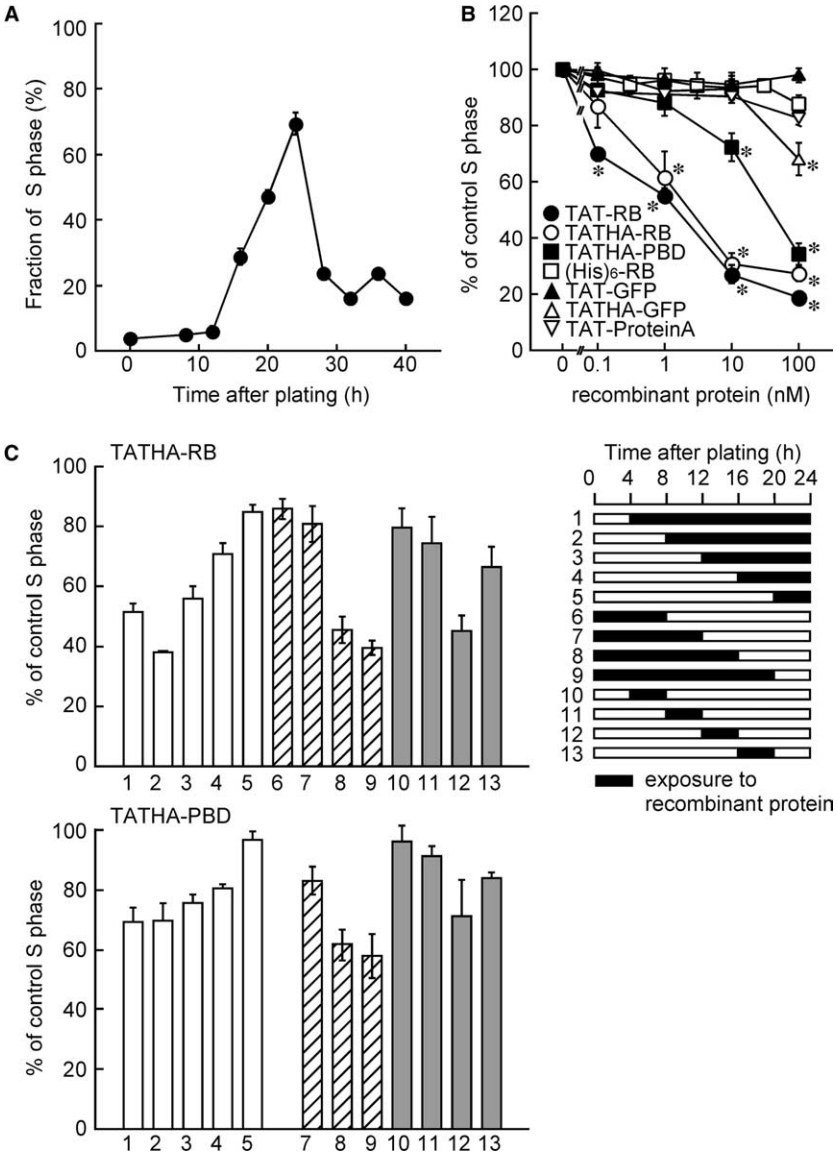


Fig. 5. A reversible inhibition of the cell cycle progression by RB and PBD in BAECs. (A) The time course of progression to the S phase in the control cells after replating at a confluent density of 25%. The data are means ± S.D. ($n = 3$). (B) A summary of the concentration-dependent effects of recombinant proteins on the S phase progression as evaluated at 24 h. The recombinant proteins were applied at time 0. The S phase progression was expressed as a percentage of that obtained in the control. The data are means ± S.D. ($n = 3$). *, $P < 0.05$ vs. the control (100%). (C) Time-specific effect of 100 nM TAHA-RB and TATHA-PBD on the cell cycle progression as evaluated at 24 h. The cells were exposed to the recombinant proteins according to the protocol shown in the key. The data are means ± S.D. ($n = 3$).

When applied to the cells by 16 h after replating, TATHA-RB markedly inhibited the S phase progression (Fig. 5C, protocols 1–3). When applied at 16 h and 20 h, TATHA-RB was less effective in inhibiting the S phase progression (Fig. 5C, protocols 4 and 5). On the other hand, when removed after 16 h, TATHA-RB markedly inhibited the S phase progression (Fig. 5C, protocols 8 and 9). When removed by 12 h, it was less effective (Fig. 5C, protocols 6 and 7). In protocols 10–13, the 4-h exposure during 12–16 h was found to most effectively inhibit S phase progression (Fig. 5C, protocol 12). The similar but weaker time-specific inhibition of the S phase progression was observed with TATHA-PBD. As a result, the period 12–16, corresponding to the $G_1$–S transition phase, was the most critical period that required the RhoA and Rac1/Cdc42 activity for the S phase progression.

## 4. Discussion

The present study provides biochemical and functional evidence that Tat PTD-mediated protein transduction is reversible, and that intracellularly transduced proteins are extruded from the cells when extracellular proteins were removed. First, an immunoblot analysis detected not only a decline in the amount of the introduced proteins in the cell extract but also an increase in the amount of the extracellular proteins. There is a possibility that the decline of Tat-tagged proteins was merely due to wash-out of any proteins attached to the extracellular surface but not due to extrusion of the intracellularly transduced protein [10]. However, this possibility was ruled out by the observations obtained with the cells treated with trypsin. Second, a flow cytometric analysis demonstrated that the GFP fluorescence

intensity declined over time after the removal of extracellular PTD-tagged GFP. Finally, a time-specific treatment with TATHA-RB and TATHA-PBD inhibited the S phase progression in a time-specific manner. The reversibility of protein transduction thus suggests that PTD-mediated protein delivery is a consequence of equilibrium between intracellular and extracellular proteins, which is consistent with a previous report [28]. It is thus also possible to introduce proteins in a quantitative manner with Tat PTD-mediated protein transduction.

The extrusion of the transduced proteins was found to be sensitive to low temperatures. However, it was resistant to metabolic inhibition and proteasome inhibition. Furthermore, the kinetics of the extrusion process was dependent on the size of the molecule. The heparan sulfate proteoglycan was reported to serve as a receptor for entry of Tat PTD [10,14]. Such a receptor is absent on the cytoplasmic surface of the plasma membrane. Our observations thus suggest that such extrusion is due to the energy-independent passive diffusion across the plasma membrane as a consequence of the equilibration shift toward an outflow-dominated state. A low temperature is considered to reduce the membrane fluidity, thereby impeding protein extrusion.

The precise mechanism of protein uptake mediated by cell-penetrating peptides still remains to be elucidated. Three modes of uptake have been proposed at present; a direct penetration-mediated uptake, an inverted micelle-mediated uptake and a endocytosis/pinocytosis-mediated uptake [29]. The recent publications suggest that the majority of uptake is mediated by endocytosis or pinocytosis, followed by a partial release from the vesicles, although the escape from pinocytosis was reported to be an inefficient process [10–12,29]. The involvement of vesicle-driven uptake is apparently against the reversibility of protein transduction. Our findings thus support the direct penetration or inverted micelle-driven delivery. Alternatively, the uptake mechanism may differ with cargo and cell type [4,5,15,29].

Both the time-specific delivery as well as the quantitative transduction of proteins are difficult to perform with a conventional transfection of nucleotides and its resulting protein expression. Our analysis of the effects of RB and PBD on the cell cycle progression, for the first time, revealed that the $G_1$–S transition phase of the cell cycle is the critical period requiring RhoA and Rac1/Ccd42 for the S phase progression. Furthermore, the concentrations of RB and PBD that are required for the cell cycle inhibition were found to correlate to those found in endogenous proteins [30]. Our functional data thus support the reversibility and quantitativeness of Tat PTD-mediated protein transduction. Precisely, how RhoA and Rac1/Cdc42 regulate the cell cycle progression in the $G_1$–S transition phase still remains to be elucidated.

In conclusion, when performed at 37 °C, Tat PTD could deliver proteins into cells with an intact plasma membrane, in a rapid, quantitative and reversible manner. The equilibrium across the plasma membrane thus determines the extent of protein transduction. The time-specific delivery of inhibitor proteins of Rho proteins revealed a critical period requiring their activity to successfully carry out the S phase progression in BAECs. The Tat PTD-mediated protein transduction thus could be a powerful tool to investigate the time-specific and quantitative role of signaling proteins in the cell function.

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