3. U. S. Vogel *et al.*, *Nature* **335**, 90 (1988); M. Trahey *et al.*, *Science* **242**, 1697 (1988).
4. T. Pawson, *Oncogene Res.* **3**, 491 (1988).
5. B. J. Mayer, M. Hamaguchi, H. Hanafusa, *Nature* **332**, 272 (1988).
6. B. J. Mayer and H. Hanafusa, *J. Virol.*, in press.
7. B. J. Mayer, M. Hamaguchi, H. Hanafusa, *Cold Spring Harbor Symp. Quant. Biol.* **53**, 907 (1989); B. J. Mayer and H. Hanafusa, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2638 (1990).
8. G. Kimura, A. Itagaki, J. Summers, *Int. J. Cancer* **15**, 694 (1975); C. P. Marshall and H. Hanafusa, in preparation.
9. M. Matsuda, C. P. Marshall, H. Hanafusa, *J. Biol. Chem.*, in press.
10. T. Takeya and H. Hanafusa, *Cell* **32**, 881 (1983).
11. S. Kawai *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6199 (1980).
12. T. Graf and H. Beug, *Biochim. Biophys. Acta* **516**, 269 (1978).
13. B. M. Sefton *et al.*, *J. Virol.* **41**, 813 (1982); T. Hunter and B. M. Sefton, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311 (1980).
14. J. Meisenhelder, P.-G. Suh, S. G. Rhee, T. Hunter, *Cell* **57**, 1109 (1989); M. I. Wahl *et al.*, *Mol. Cell. Biol.* **9**, 2934 (1989); B. Margolis *et al.*, *Cell* **57**, 1101 (1989); M. I. Wahl, S. Nishibe, P.-S. Suh, S. G. Rhee, G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1568 (1989).
15. D. A. Kumjian, M. I. Wahl, S. G. Rhee, T. O. Daniel, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8232 (1989); B. Margolis *et al.*, *Mol. Cell. Biol.* **10**, 435 (1990).
16. J. A. Cooper, K. L. Gould, C. A. Cartwright, T. Hunter, *Science* **231**, 1431 (1986); R. Jove and H. Hanafusa, *Annu. Rev. Cell. Biol.* **3**, 31 (1987).
17. D. J. McCarley, J. T. Parsons, D. C. Benjamin, S. J. Parsons, *J. Virol.* **61**, 1927 (1987); C. A. Koch, M. Moran, I. Sadowski, T. Pawson, *Mol. Cell. Biol.* **9**, 4131 (1989).
18. Conditions for cell culture, labeling, preparation of cell lysates, and immunoprecipitation were as described (*5, 7*) with the following modifications. Cells (10^7) were labeled for 5 hours with 2.5 mCi of [^{32}P]orthophosphate (Du Pont Biotechnology Systems) in 2 ml of medium. Lysates were prepared in RIPA buffer [10 mM tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and Trasylol at 100 KIU (kallikrein inactivator units) per milliliter] containing 0.5 mM Na_3VO_4, 50 μM Na_2MoO_4, and 10 mM NaF.
19. W. M. Potts, M. Olsen, D. Boettiger, V. M. Vogt, *J. Gen. Virol.* **68**, 3177 (1987).
20. M. Hamaguchi, C. Grandori, H. Hanafusa, *Mol. Cell. Biol.* **8**, 3035 (1988).
21. S. Kawai, *J. Virol.* **34**, 772 (1980).
22. SR-3Y1 cells (5×10^6) were lysed in RIPA buffer and $p60^{v\text{-}src}$ was immunoprecipitated by anti-Src (monoclonal antibody 327) [L. A. Lipsich, A. J. Lewis, J. S. Brugge, *J. Virol.* **48**, 352 (1983)] and phosphorylated in kinase buffer [10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.2 mM ATP] at 30°C for 20 min. Immune complexes were washed with RIPA and 50 mM Hepes (pH 7.5) and 0.1% Triton X-100 and incubated in phosphatase buffer [40 mM 1,4-piperazine diethane sulfonic acid (Pipes) (pH 6.0), leupeptin at 10 μg/ml, and Trasylol at 100 KIU/ml] with 10 μg of potato acid phosphatase (Sigma) at 30°C for 30 min. Immune complexes were washed with RIPA and incubated with 0.1 μg of purified $P47^{gag\text{-}crk}$ (9). Proteins were separated on a 7.5% SDS–polyacrylamide gel. Immunoblotting was performed as described in the legend to Fig. 2.
23. C. T. Reichman and H. Hanafusa, in preparation.
24. We thank D. Boettiger, J. S. Brugge, C. Y. Kang, and Y. Matsuura for providing cell lines and baculovirus vectors; and S. A. Kornbluth, G. A. Blobel, and D. W. Sternberg for reading of this manuscript. Supported by National Cancer Institute grant CA44356; grant 2517 from Council for Tobacco Research; and (to B.J.M.) NIH training grant AI 07233 from the National Institute of Allergy and Infectious Diseases.

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Antibodies to Clathrin Inhibit Endocytosis But Not Recycling to the Trans Golgi Network in Vitro

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Mannose 6-phosphate receptors carry newly synthesized lysosomal enzymes from the trans Golgi network (TGN) to prelysosomes and then return to the TGN to carry out another round of lysosomal enzyme delivery. Although clathrin-coated vesicles mediate the export of mannose 6-phosphate receptors from the TGN, nothing is known about the transport vesicles used to carry these receptors back to the TGN. Two different in vitro assays used in this study show that an antibody that interferes with clathrin assembly blocks receptor-mediated endocytosis of transferrin, but has no effect on the recycling of the 300-kilodalton mannose 6-phosphate receptor from prelysosomes to the TGN. These results suggest that the transport of mannose 6-phosphate receptors from prelysosomes to the TGN does not involve clathrin.

NEWLY SYNTHESIZED, SOLUBLE LYsosomal enzymes undergo a unique posttranslational modification that facilitates their delivery to lysosomes (*1*). As lysosomal enzymes traverse the secretory pathway, their N-linked oligosaccharides acquire one or two mannose 6-phosphate (man6P) residues. The presence of man6P enables lysosomal enzymes to bind to man6P receptors in the TGN; the receptor-ligand complexes are then thought to be carried in clathrin-coated vesicles to prelysosomes. The acidic interior of prelysosomes (also termed "late endosomes") triggers the release of the enzymes from man6P receptors, and lysosomal enzymes later appear in lysosomes. Meanwhile, man6P receptors are carried back to the TGN to complete the transport cycle. A small fraction of man6P receptors are also present at the cell surface, where they can bind extracellular lysosomal enzymes and deliver them to lysosomes by conventional receptor-mediated endocytosis.

We have recently reconstituted the recycling of the 300-kD man6P receptor from late endosomes to the TGN in a cell-free system (*2*). The assay takes advantage of the localization of sialyltransferase to the trans Golgi and TGN (*3*) and utilizes a mutant cell line [Chinese hamster ovary (CHO) clone 1021] in which glycoproteins are not sialylated (*4*). Man6P receptors (metabolically labeled with [^{35}S]methionine), present in late endosomes in a mutant cell extract, acquire sialic acid residues when they are transported to the TGN of wild-type Golgi complexes, which are present in reaction mixtures. The acquisition of sialic acid by man6P receptors in this system reflects a vesicular transport process, since it is dependent on time, temperature, adenosine triphosphate (ATP), and cytosol and also requires guanosine triphosphate (GTP) hydrolysis (*2*). Furthermore, man6P receptors and sialyltransferase remain in sealed membrane compartments throughout the reaction, and nonspecific membrane fusion is ruled out by several criteria (*2*).

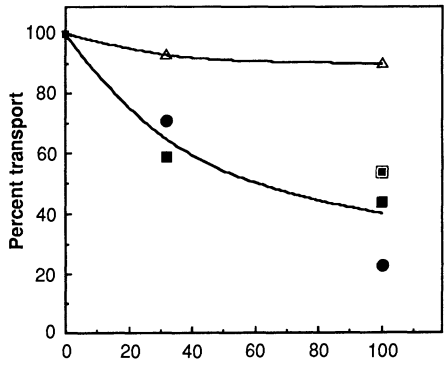

Fig. 1. Anti-clathrin IgG inhibits endocytosis but not man6P receptor recycling to the TGN in vitro. Endocytosis (closed symbols) was measured as described in Table 1; the different symbols refer to three independent endocytosis experiments and include the data presented in Table 1. Transport of 300-kD man6P receptors from endosomes to the TGN (open triangles) was assessed in three independent experiments by transport-coupled sialylation of man6P receptors (*2*) in reaction volumes of 200 μl; a typical result is shown. Endosome-TGN reactions were preincubated with antibodies for 30 min at 0°C. Samples were then warmed to 37°C for 2 hours. In control endosome-TGN reactions in the absence of antibody, 10 to 20% of total man6P receptors acquired sialic acid. Antibody preincubation had no effect on the inhibition observed for endocytosis. Data are presented as the percent transport observed relative to transport measured in the presence of a control IgG.

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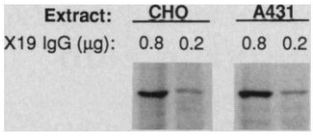

Fig. 2. Immunoprecipitation of clathrin from metabolically labeled CHO and A431 cells. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 hours and chased for 1 hour in normal growth media as described (*14*). Immunoprecipitations were carried out with ~250 μg of cell extract, and either 0.8 or 0.2 μg of anti-clathrin IgG (*14*). After 3 hours on ice, 5 μg of affinity-purified goat antibody to mouse IgG was added for an additional 15 min at room temperature. Immune complexes were adsorbed to *Staphylococcus aureus*, washed, analyzed on 6% SDS-polyacrylamide gels, and autoradiographed as described (*14*).

Nothing is known about the transport vesicles that carry man6P receptors from late endosomes to the TGN. We used a monoclonal antibody (X19) that recognizes an epitope on the clathrin heavy chain (*5*) to investigate the possible role of clathrin and clathrin-coated vesicles in man6P receptor recycling. X19 Fab fragments inhibit the assembly of clathrin baskets from purified clathrin triskelions, and intact X19 IgG (immunoglobulin G) induces the formation of large mats of clathrin aggregates (*6*). In addition, the intact antibody has been shown to inhibit both fluid phase endocytosis and receptor-mediated endocytosis when introduced into the cytoplasm of African green monkey kidney cells (*7*).

To verify that antibody X19 could inhibit a clathrin-dependent transport process in vitro, we tested the effect of X19 on transferrin endocytosis using an assay described by Smythe *et al.* (*8*). In this assay, A431 human epidermoid carcinoma cells are incubated with ^{125}I-labeled transferrin at 4°C to label cell surface transferrin receptors. Cells are then gently broken by scraping, and the "semi-intact" cells are incubated at 37°C in the presence of cytosol and ATP to permit clathrin-coated vesicle formation and endocytosis. Transferrin endocytosis is then assessed by an immunoassay that measures the acquired inaccessibility of transferrin to transferrin antibodies (*8*). In vitro endocytosis is ATP- and cytosol-dependent (*8*). Slight modifications were incorporated to make the in vitro endocytosis conditions as similar as possible to those of the man6P receptor recycling assay.

In control reactions, the extent of endocytosis and its dependence on temperature (Table 1) were essentially identical with that reported by Smythe *et al.* (*8*). In the presence of anti-clathrin X19 IgG, endocytosis was inhibited up to ~65%, relative to the amount of endocytosis observed in the presence of an equal amount of a control IgG of the same subclass.

At the highest concentration used, X19 IgG was present at a roughly fivefold molar excess relative to clathrin (*9*). Nevertheless, the inhibition of transferrin endocytosis was incomplete. A possible explanation for these findings, which is consistent with previous electron microscopy (*8*), would be that a proportion of the transferrin resided in almost completed coated pits at the beginning of the transport reaction. In this case, endocytosis would require very little clathrin assembly, making it impossible for the antibody to inhibit all endocytosis. Despite this limitation, significant inhibition was observed.

Receptor-mediated endocytosis was inhibited by clathrin-specific antibodies in a dose-dependent manner (Fig. 1, closed symbols), as would be expected from published experiments (*7*). In contrast, the same concentrations (and preparations) of X19 IgG had no effect on the recycling of the 300-kD man6P receptor from late endosomes to the TGN (Fig. 1, open triangles), relative to the amount of transport measured in the presence of an equal concentration of control IgG.

X19 IgG blocks clathrin function in African green monkey kidney cells (*7*). We have shown here that this antibody can block endocytosis in human A431 cells in semi-intact cell extracts supplemented with CHO cell cytosol. We estimate that these reactions contained at most ~20 μg of human clathrin per milliliter (from the A431 cells) and ~20 μg of CHO clathrin per milliliter (from the added cytosol) (*9*). In contrast, man6P receptor recycling assays contained a total of ~40 μg of CHO clathrin per milliliter. It was therefore important to rule out the possibility that X19 IgG did not inhibit man6P receptor recycling because it did not recognize CHO cell clathrin. For this purpose, equal amounts of extracts from metabolically labeled A431 and CHO cells were immunoprecipitated with limiting concentrations of X19 IgG and analyzed for cloth-

Table 1. Effect of anti-clathrin IgG on transferrin endocytosis by human A431 cells in vitro. Reactions were carried out as described (*8*). Briefly, after binding radiolabeled transferrin at 4°C, semi-intact cells were prepared and incubated at 31°C with ATP and cytosol to permit endocytosis. After chilling, anti-transferrin antibodies were added to bind to exposed (nonendocytosed) transferrin molecules. Membranes were then solubilized in the presence of transferrin and *Staphylococcus aureus* cells to bind antibody-tagged, accessible transferrin; inaccessible (endocytosed) transferrin (which lacked bound antibody) was quantified by counting the supernate fraction obtained after low-speed centrifugation (*8*) and is stated ±SEM for three experiments. Radioactivity in the 0°C supernatants was taken as background and was subtracted from samples incubated at 31°C to obtain counts per minute of transferrin endocytosed. The following modifications were made to make the endocytosis assay as similar as possible to the man6P receptor recycling assay. Cells grown in 10-cm dishes were scraped in the presence of 0.8 ml of reaction mix, containing protease inhibitors [aprotinin (0.425 U/ml), leupeptin (12.5 μg/ml), pepstatin (1.25 μM)], buffer (25 mM Hepes/KOH, 1.5 mM magnesium acetate, and 115 mM KCl, pH 7.2), ATP (1.25 mM), creatine phosphate (18.75 mM), creatine phosphokinase (26.25 IU/ml), 2.5 mM $MgCl_2$, and CHO cytosol (~120 μg/ml). Fifty-microliter aliquots were dispensed into microfuge tubes. One set was maintained at 0°C, a second set received control IgG (29B5, an IgG_1 directed against dinitrophenol), and the third received X19 IgG (also an IgG_1). The latter two sets were incubated at 31°C for 10 min and then returned to ice. IgG was purified as described (*5*).

Conditions	Inaccessible transferrin (cpm)	Background subtracted	Percent of control
No addition, 0°C	852 ± 113	0	
+ Control IgG, 32 μg/ml	2146 ± 181	1294	100
+ Anti-clathrin IgG, 32 μg/ml	1616 ± 7	764	59
+ Control IgG, 100 μg/ml	1643 ± 34	791	100
+ Anti-clathrin IgG, 100 μg/ml	1200 ± 29	348	44

Table 2. Effect of anti-clathrin IgG on endocytosis in broken CHO cells. Transferrin endocytosis was carried out with CHO cells expressing human transferrin receptors (*10*) in the presence of 100 μg of the indicated IgG per milliliter as described in Table 1. ATP dependence was ascertained by carrying out reactions in the presence of either 56 U/ml glycerokinase and 10 mM glycerol or an ATP regenerating system (*2*); glycerol (10 mM) was added to the control incubations and had no effect. Radioactivity (counts per minute) observed in the absence of ATP has been subtracted (*10*). Values are means ± SEM. Cells on polylysine, three experiments; cells on collagen, four experiments.

Conditions	ATP-dependent inaccessible transferrin (cpm)	Percent of control
Cells on polylysine		
+ control IgG	125 ± 12	100
+ anti-clathrin IgG	44 ± 30	35
Cells on collagen		
+ control IgG	249 ± 57	100
+ anti-clathrin IgG	161 ± 33	64

rin immunoprecipitation by SDS–polyacrylamide gel electrophoresis and autoradiography. The precipitation of clathrin by X19 IgG, from either A431 or CHO cells, was virtually indistinguishable (Fig. 2). Thus, X19 IgG appears to bind to human and Chinese hamster clathrin with similar affinity. In addition, A431 and CHO cells contain roughly equivalent amounts of clathrin.

To demonstrate unequivocally that X19 IgG could inhibit clathrin function in semi-intact CHO cells, we adapted the endocytosis assay (*8*) described above for use with CHO cells expressing human transferrin receptors (*10*). For this purpose, it was essential to grow the CHO cells on polylysine- or collagen-coated dishes to obtain semi-intact cells that could internalize ^{125}I-labeled transferrin in an ATP-dependent manner (*10*). X19 IgG inhibited the ATP-dependent endocytosis of transferrin by semi-intact CHO cells by as much as 65% when cells were scraped from polylysine-coated dishes (Table 2). Somewhat less inhibition (36%) was observed if cells were scraped from collagen-coated dishes; this is likely to reflect a difference in antibody accessibility to the cytoplasmic components in each type of broken cell. In summary, these data show that X19 IgG inhibits receptor-mediated endocytosis in broken CHO cells to a similar extent as that observed in broken human A431 cells (Table 1) or intact monkey kidney cells (*7*).

Clathrin-coated vesicles mediate fluid phase and adsorptive endocytosis. Clathrin is also thought to function at the TGN, in the diversion of lysosomal enzymes and secretory storage granule content away from constitutively secreted and membrane proteins bound for the cell surface (*11*). We have used antibodies that disrupt clathrin assembly, in conjunction with two different in vitro assays, to show that clathrin-coated vesicles appear not to carry man6P receptors from prelysosomes back to the TGN. This represents the first direct indication that in a transport cycle in which clathrin mediates the forward reaction (TGN export), another type of transport vesicle may be utilized for the reverse transport step (TGN import). In addition, since the transport of proteins from late endosomes to the TGN is a selective process (*2*), these data rule out a model in which the protein, clathrin, coats all transport vesicles involved in selective intracellular transport steps (*11*).

Our findings spotlight an interesting problem, namely, how clathrin-coated vesicle components recognize and capture man6P receptors at the TGN and not in prelysosomes. It has been proposed (*12*) that Golgi-specific "adaptin" proteins (*13*) sequester man6P receptors into clathrin-coated pits at the TGN. If this model is correct, our results imply that the adaptins (or adaptin-like proteins) must somehow be released from man6P receptors, either concurrent with the uncoating of these transport vesicles or after delivery of man6P receptors to their target organelle. In addition to proteins that accomplish cargo recognition, transport vesicles must also possess proteins that specify their organelle targets. Identification of the proteins that recognize man6P receptors in prelysosomes and direct them to the TGN represents an important challenge for the future.

REFERENCES AND NOTES

1. For review, see S. Kornfeld and I. Mellman, *Annu. Rev. Cell Biol.* **5**, 483 (1989).
2. Y. Goda and S. R. Pfeffer, *Cell* **55**, 309 (1988).
3. J. Roth, D. J. Taatjes, J. M. Lucocq, J. Weinstein, J. C. Paulson, *ibid.* **43**, 287 (1985).
4. E. B. Briles, E. Li, S. Kornfeld, *J. Biol. Chem.* **252**, 1107 (1977); S. L. Deutscher, N. Nuwayhid, P. Stanley, E. B. Briles, C. B. Hirschberg, *Cell* **39**, 295 (1984).
5. F. M. Brodsky, *J. Cell Biol.* **101**, 2047 (1985).
6. G. S. Blank and F. M. Brodsky, *EMBO J.* **5**, 2087 (1986).
7. S. J. Doxsey, F. M. Brodsky, G. S. Blank, A. Helenius, *Cell* **50**, 453 (1987).
8. E. Smythe, M. Pypaert, J. Lucocq, G. Warren, *J. Cell Biol.* **108**, 843 (1989).
9. Clathrin was estimated to be ~1% of the total protein as calculated in (*7*).
10. CHO cells expressing human transferrin receptors were grown in α–minimum essential medium containing 10% fetal bovine serum and geneticin at 0.8 mg/ml. For endocytosis experiments, culture dishes were precoated at 20°C with rat tail collagen (type I, 0.1 mg/ml in 0.1 M HOAc) overnight, or with polylysine at 10 μg/ml for 1 hour. Dishes were washed twice before use; cells were used at confluency, 2 days after plating. Endocytosis was carried out as described in Table 1. A431 cell endocytosis has been shown to be ~50 to 75% ATP-dependent (*8*). Under the above conditions, CHO cell endocytosis was ~35% ATP-dependent. Since as few as 5% residual intact cells will generate a significant (but ATP-independent) endocytosis signal, ATP-independent radioactivity (counts per minute) was subtracted from the CHO cell endocytosis values presented here.
11. F. M. Brodsky, *Science* **242**, 1396 (1988); S. R. Pfeffer and J. E. Rothman, *Annu. Rev. Biochem.* **56**, 829 (1987); T. L. Burgess and R. B. Kelly, *Annu. Rev. Cell Biol.* **3**, 243 (1987); G. Griffiths and K. Simons, *Science* **234**, 438 (1986).
12. B. M. F. Pearse, *EMBO J.* 7, 3331 (1988).
13. S. Ahle, A. Mann, U. Eichelsbacher, E. Ungewickell, *ibid.*, p. 919; M. S. Robinson, *J. Cell Biol.* **104**, 887 (1987).

14. S. R. Pfeffer, *J. Cell Biol.* **105**, 229 (1987).
15. We thank L. Herzenberg and M. Snider for providing antibody 29B5 and CHO cells expressing human transferrin receptors, respectively, and S. Schmid for the invaluable suggestion of using polylysine for CHO cell endocytosis in vitro. Supported by NIH grant DK 37332 and a March of Dimes Basil O'Connor Scholar Award (5-635) to S.R.P. and NIH grant GM 38093 and funds from the Pew Memorial Trust to F.M.B. R.K.D. was supported by NIH grants GM 34297 and GM 34042 while on sabbatical with S.R.P.; Y.G. is a Markey predoctoral fellow.

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Molecular Localization of the Transforming and Secretory Properties of PDGF A and PDGF B

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Human platelet-derived growth factor (PDGF) is a connective tissue cell mitogen comprised of two related chains encoded by distinct genes. The B chain is the homolog of the v-*sis* oncogene product. Properties that distinguish these ligands include greater transforming potency of the B chain and more efficient secretion of the A chain. By a strategy involving the generation of PDGF A and B chimeras, these properties were mapped to distinct domains of the respective molecules. Increased transforming efficiency segregated with the ability to activate both α and β PDGF receptors. These findings genetically map PDGF B residues 105 to 144 as responsible for conformational alterations critical to β PDGF receptor interaction and provide a mechanistic basis for the greater transforming potency of the PDGF B chain.

HUMAN PLATELET-DERIVED GROWTH factor (PDGF) is a major mitogen for cells of connective tissue origin that is involved in development and wound healing (*1*). Abnormal expression of this growth factor has also been implicated in a variety of pathologic states including cancer (*2*). PDGF is a disulfide-linked dimer consisting of two related polypeptide chains, designated A and B, that are products of different genes. The gene encoding the human PDGF B chain is the normal counterpart of the v-*sis* oncogene (*3*). PDGF A and B chains are approximately 40% related (*4*) and contain eight conserved cysteine residues (*5, 6*). PDGF A and B chains can form homodimers as well as the AB heterodimer,

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