**INTRODUCTION**

The Golgi apparatus plays a central role in the endomembrane system of eukaryotic cells (Farquhar, 1985; Mellman and Simons, 1992). In mammalian cells the organelle usually seems to be divided into three functionally defined areas: the cis-Golgi network, which receives vesicular traffic from the endoplasmic reticulum; the stacked cis, medial and trans cisternae, where sequential oligosaccharide modifications are carried out; and the trans-Golgi network, where late post-translational modifications occur and where sorting of proteins to various post-Golgi destinations is thought to be achieved (Griffiths and Simons, 1986; Sossin et al., 1990).

The exact nature of the Golgi in the yeast *Saccharomyces cerevisiae* is uncertain. It is clear that yeast cells possess a functional equivalent of the mammalian Golgi, where glycoproteins are modified, proproteins are proteolytically processed and proteins are sorted to either the cell surface or the vacuole; and that these various post-translational modifications occur in separate cellular compartments (Cunningham and Wickner, 1989; Franzusoff and Schekman, 1989; Graham and Emr, 1991). However, it has proved difficult to visualise any morphological equivalent of the mammalian stack of cisternae in budding yeast (but see Preuss et al., 1992). In order to exploit fully the progress that has been made in the identification and characterisation of specific proteins involved in the operation of the yeast endomembrane system it is essential to characterise the yeast Golgi: to this end we have developed a procedure for the isolation of one clearly defined compartment of this pathway organelles while being significantly enriched for two other late Golgi enzymes, dipeptidylaminopeptidase A and the Kex1 carboxypeptidase. Our findings provide clear evidence for a single yeast Golgi compartment containing all three late-processing enzymes, which is likely to be the functional equivalent in yeast of the mammalian trans-Golgi network.

**SUMMARY**

One of the Golgi compartments of *Saccharomyces cerevisiae* is defined by the presence of a specific endoprotease, Kex2p, which cleaves precursor polypeptides at pairs of basic residues. We have used antibodies directed against the cytoplasmically disposed C-terminal domain of Kex2p to develop an immuno-affinity procedure for the isolation of Kex2p-containing organelles. The method gives a high yield of sealed organelles that are essentially free of contamination from other secretory pathway organelles while being significantly enriched for two other late Golgi enzymes, dipeptidylaminopeptidase A and the Kex1 carboxypeptidase. Our findings provide clear evidence for a single yeast Golgi compartment containing all three late-processing enzymes, which is likely to be the functional equivalent in yeast of the mammalian trans-Golgi network.

Key words: Golgi, immunoisolation, Kex2p, sec7, yeast

**MATERIALS AND METHODS**

**Strains and growth media**

*Escherichia coli* strain NMS22 (Gough and Murray, 1983) was used as the host for plasmid constructions and the production of Protein A fusion proteins; the strain pop2136 (Kusters et al., 1989) was used for the production of βGal fusion proteins. Bacteria were grown in LB medium supplemented with appropriate antibiotics.

For most of the experiments involving *Saccharomyces cerevisiae* the strains used were the haploid strain JRY188 (MATα leu2-3, 112 ura3-52 trpl his4 sir3 rme, Brake et al., 1984) or its homozygous diploid derivative NBY10. To produce this diploid strain JRY188 was first transformed with the plasmid pGAL-HO
(Herskovitz and Jensen, 1991). Transformed cells were transferred to galactose-containing medium to allow mating type interconversion. A number of single colony isolates were transferred back on to glucose-containing medium and selection for pGAL-HO was removed to allow the isolation of plasmid-free segregants. NBY10 was chosen for further study as a diploid (it did not produce α-factor, it failed to mate with tester strains of either mating type, and it sporulated at low frequency to produce four-spored ascii). In the experiment in which the distribution of epitope-tagged Mnt1p was analysed we used strain 6210-mntmyc (obtained from Dr S. Munro); and in experiments in which the effects of a sec7 mutation were analysed we used SF294-2B (MATα sec7-1; Novick et al., 1980). For experiments in which β-lactamase activity was measured the strain used was JRY188 transformed with the plasmid pYS50. This is a 2 μm-LEU2-based plasmid encoding a prepro-α-factor to β-lactamase fusion protein. Yeast strains harbouring this plasmid produce and secrete active β-lactamase: in a mid-log culture 95% of detectable β-lactamase protein and enzyme activity are in the culture supernatant (A. J. Mileham and A. Boyd, unpublished data). Yeast cultures were grown in SD medium (0.67% nitrogen base without amino acids (Difco); 2% glucose) supplemented with appropriate nutrients.

**Immunological procedures**

Antibodies that specifically recognise either the luminal N-terminal domain or the cytoplasmically disposed C-terminal domain of Kex2p were obtained using bacterially produced fusion proteins. To obtain the C-terminal domain antibody, a spa-KEX2 gene fusion encoding a protein consisting of the 100 C-terminal amino acids of Kex2p fused to a portion of staphylococcal Protein A was constructed by insertion of a 1.25 kb EcoRI/Rsal fragment from the KEX2-containing plasmid pGA714 (obtained from Dr G. Ammerer) into similarly digested pKpra (Zueco and Boyd, 1992). *E. coli* cells harbouring this plasmid produced a fusion protein with a relative molecular mass of 29,000, which was purified from bacterial lysates by chromatography on IgG-Sepharose (Lowe-nadler et al., 1986). For the N-terminal domain antibody, a spa-KEX2 gene fusion encoding a protein containing amino acids 119-614 of Kex2p was constructed by inserting a DNA fragment derived from the HindIII-PvuII region of the KEX2 gene into pKpra. *E. coli* cells harbouring this plasmid produced a fusion protein with a relative molecular mass of 75,000, which was purified from bacterial lysates by isolation of insoluble inclusion bodies (Marston and Hartley, 1990) followed by preparative SDS-PAGE. The purified fusion proteins were injected into rabbits. Antibodies that recognise the Kex2p-derived portions of the Spa-Kex2p fusion proteins were affinity-purified from immune sera using columns of Affi-gel (Bio-Rad) to which had been conjugated appropriate βGal-Kex2 fusion proteins (encoded by lacZ-KEX2 gene fusions created in the pEX series of vectors (Kusters et al., 1989) and containing the same Kex2p amino acids as the corresponding Spa-Kex2p fusion protein).

**Immunosolation of membranes**

An immunoadsorbent (Kex2-ImAd) was formed by binding 500 μg of anti-Kex2p antibody (C-terminal domain) to 100 μl Pan sorbin (10% (w/v) fixed *Staphylococcus aureus* cells; Calbiochem) that had previously been incubated with Solution A (20 mM Hepes, pH 7.4, 2 mM MgCl2, 150 mM KCl) containing 10 mg/ml BSA. Binding of antibodies to the Pansorbin was achieved by incubation with the affinity-purified antibody on a rotating wheel at 4°C overnight. Antibody-coated Pansorbin was then washed twice using Solution A and once in Solution B (0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA, brought to pH 7.4 with acetic acid).

Glass bead lysates were prepared as follows: cells were harvested (by centrifugation for 5 minutes at 3,000 g) from a culture that had been grown to an A600 of 0.7, washed once in distilled water and resuspended to 100 A600 units/ml in Solution B containing protease inhibitors (4 mM phenylmethylsulphonyl fluoride (PMSF), 4 mM EDTA, 4 mM EGTA, 2 μg/ml each of pepstatin, leupeptin, chymostatin and antipain). Glass beads (40 mesh) were added to the level of the meniscus and the tubes were vortexed 4×, for 30 seconds, with 2 minute cooling intervals on ice. The lysate was collected by centrifugation through the glass beads at 3,000 g for 10 minutes, resulting in a pellet of cell debris and a supernatant of cell lysate containing all detectable Kex2p. Spheroplast lysate was prepared as follows: cells were harvested and washed as before and then resuspended in spheroplast buffer (1.4 M sorbitol, 50 mM potassium phosphate, pH 7.4, 10 mM NaN3, 40 mM β-mercaptoethanol) containing zymolysate (5 mg/ml). Cell wall digestion was achieved at 25°C, and this incubation was carried out until at least 80% of the cells present had been converted to spheroplasts (30 minutes). Spheroplasts were harvested (2 minutes at 4,000 g) and washed once in spheroplast buffer before being resuspended in 1 ml Solution B containing protease inhibitors (as above) per 100 A600 units of the original culture, and then homogenised using a Dounce homogeniser (7 strokes) on ice. The homogenate was cleared of cell debris by centrifugation (2 minutes at 4,000 g) before use, resulting in a homogenate containing all detectable cellular Kex2p.

ImAd was resuspended in yeast cell lysate that was prepared from 30 A600 units of cells.

For the recovery of Kex2p-containing membranes the ImAd was incubated with cell lysate for 3 hours at 4°C on a rotating wheel to allow gentle mixing, recovered by centrifugation for 2 minutes at 4,000 g and then washed three times in Solution B.

**Immunoblotting**

Samples were subjected to 10% SDS-PAGE (Laemmli, 1970) prior to transfer to nitrocellulose. Material bound to ImAd was analysed by resuspension in SDS sample buffer, followed by incubation in a boiling water bath for 5 minutes after which the *S. aureus* cells were removed by centrifugation prior to SDS-PAGE. Immunoblots were developed using the ECL detection system (Amersham). Primary antibodies were as described, secondary antibodies were goat anti-rabbit IgG/horseradish peroxidase conjugate and goat anti-mouse IgG/horseradish peroxidase conjugate as appropriate.

**Enzyme assays**

NADPH cytochrome c reductase, a marker for the presence of ER membranes, was assayed by following the sample’s ability to reduce NADPH, as described by Yasukochi and Masters (1976). Carboxypeptidase Y (CPY) and dipeptidyl aminopeptidase (DPA P) B activities were used to detect the presence of vacuolar contents and membranes, respectively. To determine CPY activity the sample’s activity against the peptide substrate N-carboxyoxyphe-nyl-leucine was assayed as described by Wolf and Weiser (1977). Total DPA P activity present in a sample was determined by its activity against alanylprolyl-4-nitroanilide as described by Roberts et al. (1991). The activity of DPA P A (which is heat stable) was assayed by incubating the sample to 65°C for 15 minutes prior to its addition to the reaction mixture, and the activity of DPA P B was estimated by subtracting the amount of heat-stable DPA P activity in a sample from the total amount of DPA P activity in that same sample. GDPase activity was assayed as described by Abeijon et al. (1989) with the amount of free phosphate being determined as described by LeBel et al. (1978). Kex2 protease activity was determined as described by Fuller et al. (1989a). For enzymes other than Kex2, the amount of enzyme activity detected
in ImAd-bound material was expressed as a percentage of the total present in the cell lysate from which the material was bound. Material recovered from the same lysate using Pansorbin that had not been coated by antibody was also assayed for the presence of the various enzymes and it was found that this technique led to less than 1% of cellular activity of each of the enzymes being recovered (data not shown). The amount of Kex2 protease activity bound by ImAd was estimated by measuring the depletion of activity from the cell lysate following removal of ImAd (this approach was shown to be valid by immunoblotting material bound by ImAd, and material remaining in the cell lysate following treatment with ImAd to detect Kex2p; data not shown). This was necessary, since it was found that the presence of Pansorbin inhibited the Kex2 protease activity assay.

**Sucrose gradient fractionation**

Total cell extract was prepared from 250 $A_{600}$ units of cells by vortexing harvested cells in the presence of glass beads in 2 ml 55% sucrose (w/w) containing 10 mM MES, 2-(N-morpholino)ethane sulphonic acid, pH 6.5, for 1 minute. The extract was transferred to a centrifuge tube, and then overlaid with the following sucrose solutions (as described by Bowser and Novick, 1991): 1 ml 50%, 1 ml 47.5%, 1.5 ml 45%, 1.5 ml 42%, 1.5 ml 40%, 1 ml 37.5%, 1 ml 35%, 1 ml 30%, all containing 10 mM MES, pH 6.5. The gradients were spun at 170,000 g in a SW41 rotor (Beckman Instruments Inc.) for 16 hours and 1 ml fractions were collected from the top of the gradient for further analysis.

For determination of the latency of a soluble secretory cargo enzyme two lysates were prepared from yeast cells secreting the bacterial enzyme $\beta$-lactamase. One lysate was prepared using glass bead lysis and the other by homogenisation of spheroplasts. The activity of $\beta$-lactamase in 5 µl of each lysate (equivalent to 0.5 $A_{600}$ unit of original culture) was assayed using the substrate nitrocefin (O’Callaghan et al., 1972) in both the presence and absence of 0.1% Triton X-100 in order to determine what proportion of the enzyme was contained within membrane structures (5 mg nitrocefin was dissolved in 0.5 ml DMSO before being diluted to 0.5 mg/ml with 0.1 M sodium phosphate, pH 7.0; 50 µl of this stock nitrocefin solution was added to 900 µl 0.1 M sodium phosphate, pH 7.0, 0.8 M sorbitol; the sample was then diluted into 50 µl of Solution B, added to the assay, and the increase in $A_{490}$ was followed). The activity measured in the presence of detergent was taken as the total lysate activity (lysate prepared by glass bead lysis contained 0.12 arbitrary unit of activity ($\Delta A_{490}$/min) and that prepared by homogenisation contained 0.09 unit) and the activity measured in the absence of detergent was expressed as a percentage of this.

**Digestion with Proteinase K**

Proteinase K was added to samples to a final concentration of 50 µg/ml as indicated (Triton X-100 was added to a final concentration of 0.1% prior to this, where appropriate). The samples were incubated on ice for 60 minutes after which PMSF was added to a final concentration of 3 mM.

**RESULTS AND DISCUSSION**

**Immunoisolation of Kex2p-containing membrane vesicles**

The Kex2 protein is a type I glycoprotein with a cytoplasmically disposed domain consisting of the C-terminal 115 residues of the protein (Fuller et al., 1989a). As the basis for immunoisolation of Kex2p-containing organelles we prepared affinity-purified antibodies from a rabbit antiserum raised against a bacterially produced fusion protein incorporating the C-terminal 100 residues of Kex2p. These antibodies recognised a single polypeptide of relative molecular mass 135,000 in yeast protein extracts (Fig. 1(a), lane 1), which was identified as Kex2p. It was absent from a lysate prepared from a strain carrying a disrupted copy of the KEX2 gene and was present in elevated amounts in a lysate prepared from a strain harbouring a multicopy plasmid carrying the KEX2 gene (data not shown).

Affinity-purified anti-Kex2p antibodies were bound to
fixed *S. aureus* cells (Pansorbin) forming an immunoadsorbent (ImAd) that was used to isolate Kex2p-containing membranes from yeast cell lysates prepared using glass bead breakage. As shown in Fig. 1(a), the ImAd-bound material was found to contain Kex2p, which was not recovered when Pansorbin lacking bound antibodies was used in place of ImAd (note that the heterogeneous smear of immunoreactive material present in the bound fractions is due to the presence of protein A-derived polypeptides in the samples analysed). This recovery of Kex2p by ImAd was completely blocked by preincubation of the ImAd with the βGal-Kex2 fusion protein against which the Kex2p-specific antibodies had been affinity-purified, but not by preincubation with an unrelated βGal fusion protein (data not shown). The membrane association of the ImAd-bound Kex2p was confirmed in a protease accessibility experiment (Fig. 1(b)). For this experiment it was necessary to use an antibody directed against the luminal domain of Kex2p. This antibody revealed the presence in cell lysates of an additional, truncated form of the protein (relative molecular mass 100,000) as has been previously reported (Fuller et al., 1989a). Addition of proteinase K to ImAd-bound material caused limited digestion of Kex2p, resulting in its conversion to a polypeptide of relative molecular mass 100,000, consistent with the removal of the C-terminal domain from the protein, whereas in the presence of detergent the proteinase K completely digested the protein. From these results, we conclude that the Kex2p recovered using ImAd is contained within sealed membrane vesicles, orientated with their N-terminal domains inside, protected from externally added protease and with their C-terminal domains exposed, as found within the cell.

Under optimised binding conditions 80% of total lysate Kex2 protease activity was removed from cell lysate by ImAd (Fig. 2), whereas essentially none was bound by control Pansorbin (data not shown). Although it was not possible to assay Kex2 protease activity accurately in the bound fraction due to interference by Pansorbin, the presence of this proportion of lysate Kex2p in ImAd-bound material was confirmed by immunoblot analysis (not shown). As well as being recovered in high yield, the Kex2p-containing membranes were substantially free of activities associated with marker enzymes of other organelles (Fig. 2(a)). Thus, ImAd-bound membranes containing 80% of cellular Kex2p contained less than 1% of the endoplasmic reticulum (ER) membrane marker enzyme NADPH-cytochrome c oxidoreductase (Roberts et al., 1991) and less than 0.5% of the activities associated with two vacuolar enzymes (Roberts et al., 1991): the integral vacuolar membrane protein dipeptidyl aminopeptidase B (DPAP B) and the soluble vacuolar enzyme CPY.

It is generally thought that Kex2p resides in a compart-
ment of the yeast Golgi that lies distal to those active in the addition of mannose residues to glycoprotein oligosaccharides. In agreement with this is the finding that membranes containing GDPase activity (a marker for all sub-compartments active in mannosyltransfer reactions) are separable on density gradients from Kex2p-containing membranes (Bowser and Novick, 1991; and see Fig. 4a). Immunoisolated membranes were found to lack GDPase activity (Fig. 2a), consistent with the notion that our immunoisolation procedure leads to the recovery of a single Golgi compartment.

**Co-recovery of Kex1p and DPAP A with Kex2p**

The peptides released from the pro-α-factor precursor following Kex2 cleavage are further processed by two other proteases (Julius et al., 1984; Fuller et al., 1988): the STE13 gene product, dipeptidyl aminopeptidase A (DPAP A) (Julius et al., 1983), and a carboxypeptidase encoded by the KEX1 gene (Kex1p) (Dmochowska et al., 1987). We anticipated that membranes isolated by virtue of their Kex2p content might also contain these two proteins, but in fact found that an ImAd-bound fraction containing 80% of cellular Kex2p contained less than 5% of lysate DPAP A activity. Since glass bead breakage of yeast cells had been used to generate the lysate for this experiment we tested the idea that extensive fragmentation of the Kex2p-containing organelle might be taking place, resulting in physical separation of the two proteins into separate vesicles. Accordingly, we turned to the use of a potentially more gentle lysis method, homogenisation of spheroplasts. The integrity of secretory pathway organelles in lysates prepared by the two methods was assessed by determining the degree of latency of a soluble secretory cargo protein. As shown in Fig. 2b, the proportion of a soluble cargo protein remaining latent following cell lysis was greatly increased by the use of spheroplast homogenisation, suggesting that disruption of organelles was much reduced. Using these lysis conditions it was still possible to recover 80% of the Kex2p-containing membranes from a cell lysate without compromising the purity of the fraction as determined by assays of the ER and vacuolar membrane marker enzymes, but the ImAd-bound material now contained 25% of lysate DPAP A activity (Fig. 2c). Furthermore, when prepared using this method, the immunoisolated material was found to contain a large proportion of the cellular content of Kex1p as revealed by immunoblotting (Fig. 3). In contrast to its content of DPAP A and Kex1p, the material immunoisolated from a homogenate did not contain GDPase activity (Fig. 2c), indicating no co-recovery of other Golgi membranes. This conclusion gains support from an experiment in which immunoisolation was performed from a lysate of yeast cells producing an epitope-tagged version of the MNT1-encoded Golgi α(1-2) mannosyltransferase (Fig. 3b). This protein is thought to reside in a medial compartment of the Golgi (Graham and Emr, 1991; Nakajima and Ballou, 1975), and was found to be absent from immunoisolated Kex2p-containing membranes (Fig. 3b). From these results we conclude that we have isolated a single Golgi compartment on the basis of its content of Kex2p and that this also contains the other two Golgi late-processing proteases, DPAP A and Kex1p.

We have been unable to use our immunoisolation procedure to recover any more than 25% of cellular DPAP A in Kex2p-containing organelles. It is of course possible that Kex2p and DPAP A are based in the same organelle and spend a proportion of their time in different retrieval or recycling pathways, the existence of which have been suggested by various workers (Franzusoff et al., 1991; Seeger and Payne, 1992): if Kex2p and DPAP A were recycled from secretory vesicles to the Golgi in different populations of retrieval vesicles, then this would go some way towards explaining our results. A second possible explanation arises from the idea that these proteins might be organised within the plane of the organelar membrane to form homogeneous domains or patches of proteins. If this were the case then our results would be explained by assuming that Kex2p and DPAP A reside in different domains that are completely separated by glass bead lysis but not by gentle homogenisation of spheroplasts. Whilst we cannot rule out the explanation that DPAP A resides, not in the same compartment as Kex2p, but in a physically associated compartment that is specifically recovered along with that containing Kex2p, we currently favour some combination of the above explanations in which the Kex2p-compartment is the last stable compartment of the Golgi.
Perturbation of Golgi organisation in a sec7 mutant

We were interested to see whether physiological conditions could be found under which the nature of the Kex2p-containing compartment was altered significantly. Morphological evidence suggests that the organisation of the Golgi is altered in sec7 mutant cells at the restrictive temperature (Novick et al., 1980). In order to see whether these changes result in a loss of the ability to separate membranes containing Kex2p from those containing GDPase, we attempted immunoisolation from such cells. Fig. 4 shows the results of sucrose density gradient analysis of membranes isolated from a sec7 mutant at the permissive (25°C) and restrictive (37°C) temperatures. Following growth at the permissive temperature, Kex2 protease and GDPase activities were clearly separated on the gradient (Fig. 4(b)), whereas when cells had been exposed to the restrictive temperature, GDPase activity shifted down the gradient to overlap extensively with the Kex2p distribution (Fig. 4(c)). We found that it was not possible to use ImAd to isolate Kex2p-containing membranes directly from lysates prepared from sec7 cells grown at 37°C, since most of the cellular Kex2p was now present in the pellet of the clearing spin performed prior to immunoisolation. We therefore immunoisolated Kex2p-containing membranes from gradient fractions spanning the peak of Kex2 activity. As shown in Fig. 4(d), the membranes immunoisolated from cells grown at the permissive temperature did not contain any significant GDPase activity, whereas up to 30% of the GDPase activity present in the relevant gradient fractions of membranes prepared from cells grown at the restrictive temperature was recovered along with Kex2p. The ability of ImAd to co-recover GDPase and Kex2p from sec7 mutant cells exposed to the restrictive temperature is consistent with the idea that Golgi organisation changes under these conditions: it is possible that a partial coalescence or mixing of the two compartments occurs, perhaps due to a breakdown in retention or retrieval systems; or, alternatively, the two compartments may remain intact but become more tightly associated with one another. Further analysis of the nature of the organelles immunoisolated from sec7 cells may help to clarify this point.

CONCLUSIONS

Our results extend and strengthen current views of Golgi organisation in yeast cells. By investigating the post-translational modifications carried by secretory cargo proteins accumulated during arrest of a sec18 mutant, Graham and...
Emr (1991) were led to conclude that the yeast Golgi is divided into a minimum of three subcompartments: a cis compartment containing α(1-6) mannosyltransferase; a medial compartment containing Mnn1p-dependent α(1-3) mannosyltransferase; and a trans compartment containing Kex2p. This placement of Kex2p in a separate post-ER compartment was consistent with earlier work, which had demonstrated physical separation of organelles containing either α(1-3) mannosyltransferase or GDPase activity from those containing Kex2p (Cunningham and Wickner, 1989; Bowser and Novick, 1991). Thus, all of the evidence available hitherto has placed the Kex2p-containing compartment near the ER-distal limit of the yeast Golgi. Our data strengthen this view, since they remove any need to postulate the existence of a subsequent compartment: all known late-processing enzymes (Kex2p, DPAP A and Kex1p) seem to be present in this single organelle. Presumably, this organelle is that which has been detected by immunofluorescence studies of the localisation of both Kex2p (Redding et al., 1991) and Kex1p (Cooper and Bussey, 1992). Analogous processing proteases in mammalian cells are associated with the trans-Golgi network, which is also thought to be the site at which various classes of proteins are sorted (Griffiths and Simons, 1986; Sossin et al., 1990). Since it is clear that in yeast cells both secretory and vacuolar cargo traverse the Kex2p compartment (Graham and Emr, 1991), we are led to the conclusion that the organelle we have isolated may be the functional equivalent of the trans-Golgi network in that it is the site at which sorting occurs of proteins destined for the cell surface and the vacuole. Immunossociation has proved to be an important technique in cell biology, contributing to the understanding of both structure and function of organelles (Franzusoff et al., 1992; Salamero et al., 1990). We envisage that the availability of our immunossoiation procedure will pave the way for further characterisation of the molecular composition of the Kex2p compartment and contriute to the development of cell-free assays of membrane traffic events in this area of the yeast secretory pathway.

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