EFFECTS OF MONENSIN ON THE PROCESSING AND INTRACELLULAR TRANSPORT OF INFLUENZA VIRUS HAEMAGGLUTININ IN INFECTED MDCK CELLS

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SUMMARY

The role of the Golgi complex in the intracellular transport of influenza virus haemagglutinin in infected MDCK cell monolayers has been investigated using monensin, a carboxylic ionophore known to disrupt the functioning of this organelle in other cell types. In untreated cells metabolically labelled 5 h post-infection with $[^{35}S]$methionine haemagglutinin was first seen in core glycosylated form, which was sensitive to the enzyme endo-$
\beta$-N-acetylglucosaminidase H (endo H). After approximately 20 min this form was converted into a terminally glycosylated, endo H-resistant form. In the presence of monensin core glycosylation of haemagglutinin was not affected, but terminal glycosylation was interrupted. Two new forms of haemagglutinin were observed, both of which were smaller than the core glycosylated form. Of these, the larger was endo H-sensitive while the smaller was endo H-resistant. These new (and uncharacterized) forms of haemagglutinin are likely to be intermediates in the normal process of terminal glycosylation, which are revealed as a result of the inhibition by monensin of the transport of haemagglutinin through the stack of Golgi cisternae.

In untreated cells 85% of the pulse-labelled haemagglutinin had reached the plasma membrane after 90 min of chase, as revealed by its sensitivity to externally applied trypsin. In monensin-treated cells, on the other hand, only 55% of the haemagglutinin had reached the plasma membrane after 90 min of chase, while 94% had arrived there after 180 min of chase.

At 5 h post-infection the density of envelope proteins detected at the apical surface of the monolayer by immunofluorescence microscopy was greatly reduced by monensin treatment. Budding of virions from the apical surface of the monolayer at 4 and 7 h post-infection was also reduced, and the normal Golgi complexes were replaced by distended vacuoles that appeared to contain poorly preserved virions.

INTRODUCTION

Many membrane proteins are co-translationally inserted into the membrane of the rough endoplasmic reticulum (Katz et al. 1977; Rothman & Lodish, 1977) and then transported, via the Golgi complex, to their final destinations within the cell (Rothman & Fine, 1980; Bergmann, Tokuyasu & Singer, 1981; Green et al. 1981). Since a cell may contain several distinct membranes, each with characteristic proteins, newly synthesized proteins must be segregated at some point during intracellular transport for delivery to different organelles. Although little information is available about the mechanism of segregation, it is widely believed that the Golgi complex plays a crucial role in directing intracellular protein transport (Tartakoff, 1980).

Ion-transporting epithelia present a particularly interesting example of this
problem. Here functional asymmetry depends upon the differential localization of proteins in the apical and basolateral surfaces of the epithelial cell layer. In a cultured epithelial cell line (MDCK), for example, the enzyme aminopeptidase is found exclusively in the apical domain of the plasma membrane, while Na⁺,K⁺-ATPase is found only in the basolateral domain (Louvard, 1980).

The discovery that enveloped viruses such as influenza and vesicular stomatitis virus (VSV) bud asymmetrically from MDCK cell monolayers (Rodriguez Boulan & Sabatini, 1978) has provided an attractive model system in which to study the polarized insertion of plasma membrane proteins. The genomes of these viruses code for only a small number of proteins and host cell mechanisms must therefore be involved in virus assembly. Before the onset of budding the envelope proteins are localized in the surface of the monolayer from which the virus will bud (Rodriguez Boulan & Pendergast, 1980), which indicates that they play an important part in directing the budding process. Consequently, a study of the intracellular transport of these proteins should provide important insights into the mechanisms underlying the biogenesis of cell polarity in epithelia.

Influenza virus, which buds only from the apical surface of MDCK cell monolayers, has two envelope proteins, haemagglutinin and neuraminidase. Of these, haemagglutinin is the more thoroughly characterized (Wilson, Skehel & Wiley, 1981). In this study the role of the Golgi complex in the intracellular transport of haemagglutinin in MDCK cells has been investigated using monensin, a carboxylic ionophore known to disrupt the functioning of this organelle in other cell types (Tartakoff & Vassalli, 1979).

MATERIALS AND METHODS

Cells and virus

MDCK cells were grown as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% newborn calf serum, non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C in an atmosphere of 5% CO₂ in air. All cells, media and supplements were supplied by Flow Laboratories, Irvine, Ayrshire.

The WSN (H1N1) strain of influenza virus was grown in fertile hen eggs and assayed by plaquing on MDCK cell monolayers essentially as described by Roth, Fitzpatrick & Compans (1979). Stocks of virus in allantoic fluid were stored in liquid nitrogen.

Infection

MDCK cells were grown until just confluent. Monolayers were washed twice with 2 ml of phosphate-buffered saline (PBS) (pH 7-4), containing 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, and infected with 10–20 plaque-forming units of virus/cell in 1 ml allantoic fluid for 1 h at 37°C. The influenza virus inoculum was aspirated at the end of this absorption period and EMEM containing 2% newborn calf serum was added as a maintenance medium. Monensin (Calbiochem-Behring, Bishops Stortford, Herts) was present at 1 μM in the maintenance medium where appropriate.

Labelling with [³⁵S]methionine

MDCK cell monolayers on 3 cm plastic dishes, 5 h post-infection, were washed twice with 2 ml of EMEM lacking methionine and then labelled, usually for 10 min, with 0.5 ml of the same medium containing 10 μCi/ml [³⁵S]methionine (Amersham International, Amersham, Bucks). In some
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experiments further labelling was prevented and incubation continued by the addition of 2 ml EMEM containing 10 times the normal level of unlabelled methionine (1.5 mg/ml). To terminate the incubation samples were washed three times with 2 ml of ice-cold PBS. The cells were disrupted and cellular DNA was digested by the addition of 10 µl of 0.2% sodium dodecyl sulphate (SDS) in 5 mM-Tris-HCl (pH 8.8) followed by 2 µl of 10 mg/ml DNase I (Sigma, Poole, Dorset). Cells were scraped from the dishes with a rubber policeman.

Treatment with endo-β-N-acetylglucosaminidase H (endo H)

A sample (10 µl) of cell lysate was taken, 10 µl 2% SDS was added and the mixture boiled for 2 min. After cooling, 200 µl of 100 mM-sodium citrate (pH 5.6) containing 0.4 mU endo H (Miles, Elkhart, Indiana, U.S.A.) and 40 µg/ml phenylmethylsulphonyl fluoride (Sigma, Poole, Dorset) was added and the sample was incubated at 37°C for 15 h. Proteins were precipitated by addition of 200 µl ice-cold acetone and resuspended in 50 µl sample buffer (see below).

SDS/polyacrylamide gel electrophoresis

Samples of cell lysate were prepared for gel electrophoresis by addition of 3 vol. of sample buffer containing 200 mM-Tris-HCl (pH 6.8), 2% SDS, 500 mM-mannose, 20 mM-EDTA, 5% 2-mercaptoethanol and 0.02% bromophenol blue as a tracking dye. Samples were boiled for 2 min and cooled to room temperature before being applied to a 1 mm thick 15% (w/v) polyacrylamide gel in 0.1% SDS with a 3% stacking gel. The gel running buffer was Tris-glycine (pH 8.3) containing 0.1% SDS. After running, the gel was fixed for 15 min at room temperature in 10% trichloroacetic acid and then immersed for 1 h in ENHANCE (New England Nuclear, Dreieich, F.R.G.). The gel was washed for 15 min in water, left for 15 min in 35% (v/v) methanol and then dried at approximately 70°C under reduced pressure. Finally, it was exposed to X-ray film (Fuji, Tokyo, Japan) at −20°C, usually for 1 week.

Indirect immunofluorescence microscopy

An antiserum was raised in mice against purified whole influenza virus. This antiserum immuno-precipitated only the viral envelope proteins, haemagglutinin and neuraminidase (data not shown).

MDCK cells were grown as monolayers on glass coverslips in 3 cm plastic dishes and infected when just confluent. The cells, at 5 h post-infection, were washed three times with 2 ml of PBS and fixed with 1 ml of a 4% (w/v) solution of formaldehyde in PBS (freshly prepared from paraformaldehyde) for 20 min at room temperature. They were then washed three times with PBS and quenched with 1 ml of ammonium chloride solution (50 mM in PBS) for 10 min at room temperature. The cells were then washed three times with PBS and the coverslips were inverted onto 20 µl samples of immune or non-immune serum, each diluted 10-fold with PBS. The cells were exposed to the sera in a moist chamber for 20 min at room temperature. They were then washed four times, for 5 min each time, with PBS and exposed as above to fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Miles-Yeda, Rehovot, Israel; F/P ratio 4:0) diluted 10-fold with PBS and again washed four times with PBS. Coverslips were mounted in 50% glycerol in water and examined by darkground fluorescence using a Leitz Ortholux UV microscope equipped with excitation/suppression filters appropriate to fluorescein.

Electron microscopy

Influenza virus-infected MDCK cell monolayers on 3 cm plastic dishes were fixed with 2.5% glutaraldehyde in 0.1 M-sodium cacodylate (pH 7.2-7.4) for 30 min at 4°C, post-fixed for 15 min at 4°C in 2% osmium tetroxide, dehydrated in 70% methanol (10 min) followed by 100% methanol (3 x 10 min) and embedded in Araldite resin. Thin sections (80 nm) were cut on a Reichert Om U3 ultramicrotome, mounted on 400-mesh copper grids, stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

RESULTS

Glycosylation of haemagglutinin in untreated monolayers

At the end of the pulse-labelling period (Fig. 1) haemagglutinin was present in a
Fig. 1. Time-course of terminal glycosylation of haemagglutinin in influenza virus-infected MDCK cells. Cells were labelled 5 h post-infection with \(^{[35}S\)methionine (10 \(\mu\)Ci/ml) for 15 min and chased in the presence of excess unlabelled methionine for 0(a), 5(b), 10(c), 15(d), 20(e) and 30(f) min. Proteins were separated by SDS/polyacrylamide gel electrophoresis and detected by fluorography. Bands were assigned according to Inglis & Mahy (1979): HA, haemagglutinin; NP, nucleoprotein; M, matrix protein; NS, non-structural protein.

form which will be called HAs (see below). After 5 min of chase a larger form of haemagglutinin, HAr, began to appear and after 15 min of chase all of the
haemagglutinin was in this form. The enzyme endo-β-N-acetylglucosaminidase H (endo H) is a useful tool in the analysis of glycoprotein processing. It will cleave off the mannose-rich asparagine-linked core oligosaccharides acquired in the rough endoplasmic reticulum, leaving a single N-acetylgalactosamine residue attached to each asparagine, but not the oligosaccharides produced during terminal glycosylation in the Golgi complex, which have lost some mannose residues but gained N-acetylgalactosamine, galactose, fucose and usually sialic acid (Strous & Lodish, 1980). Fig. 2 shows the result of an experiment in which the effect of endo H on the products of pulse- and pulse–chase-labelling was investigated. After pulse-labelling only, haemagglutinin was in the HAs form, which was sensitive to endo H, indicating that HAs represents core glycosylated haemagglutinin. The product of treatment of HAs with endo H is indistinguishable in size from the unglycosylated form of haemagglutinin found during labelling in the presence of tunicamycin, a glucosamine-containing antibiotic that blocks the synthesis of dolichol-linked oligosaccharides that normally act as donors in the process of core glycosylation of nascent polypeptides at asparagine residues (Heifetz, Keenan & Elbein, 1979). After a 30 min chase period haemagglutinin was in the HAr form, which was apparently resistant to endo H. Hence HAr represents terminally glycosylated haemagglutinin.

The mature form of haemagglutinin found in MDBK cells infected with influenza virus (WSN strain) has been shown to possess six oligosaccharide moieties (Nakamura, Brown & Compans, 1980). Of these, four are resistant to endo H, one is sensitive and one is partially sensitive. Hence, even the mature form of haemagglutinin should exhibit limited sensitivity to endo H. However, the consequent reduction in size is apparently too small to be detected by SDS/polyacrylamide gel electrophoresis.

The HAs–HAr shift can be used as a marker for the movement of haemagglutinin between the rough endoplasmic reticulum and the Golgi complex. If it is assumed that the first molecules of haemagglutinin to be synthesized during the pulse are also the first to move to the Golgi complex, then Fig. 1 indicates a transit time of approximately 20 min.

Glycosylation of haemagglutinin in the presence of monensin

After monensin treatment and pulse-labelling only, haemagglutinin was found as HAs (Fig. 3). After a 30 min chase period two forms of haemagglutinin were seen,

Fig. 2. Acquisition by haemagglutinin of resistance to endo H, as a result of terminal glycosylation. Influenza virus-infected MDCK cells were labelled 5 h post-infection with [35S]methionine (10 μCi/ml) for 10 min and harvested either immediately (a,b) or after a 30 min chase period in the presence of excess unlabelled methionine (c,d). Samples either received no further treatment (a,c) or were exposed to endo H (0.4 mU) for 15 h at 37 °C (b,d). The arrowhead indicates the product of endo H digestion.
Effect of monensin on the terminal glycosylation of haemagglutinin in influenza virus-infected MDCK cells. Post-infection maintenance medium contained 1 μM monensin. Cells were labelled 5 h post-infection with [35S]methionine (10 μCi/ml) for 10 min and either harvested immediately (a,b) or chased for 30 min in the presence of excess unlabelled methionine and 1 μM monensin (c,d). Samples either received no further treatment (a,c) or were exposed to endo H (0-4 mU) for 15 h at 37 °C (b,d). The arrowhead indicates the product of endo H digestion, and the arrows the forms of haemagglutinin produced during a chase in the presence of monensin.

Both of which were smaller than HAs. Of these two new forms the larger was endo H-sensitive while the smaller was endo H-resistant. HAs and HAr were not seen at all. Hence monensin has no effect on core glycosylation but does interrupt the process of terminal glycosylation.

Effect of monensin on the intracellular transport of haemagglutinin

The final orientation of haemagglutinin in the plasma membrane of the host cell is such that almost all of the protein protrudes outside the cell, while its transmembrane and cytoplasmic segments are small (Wilson et al. 1981). When haemagglutinin has reached the plasma membrane, therefore, it should be sensitive to externally applied trypsin. The results of an experiment in which this approach was used are shown in Fig. 4. Densitometer scanning of the fluorograph revealed that in untreated monolayers 85 % of the haemagglutinin was sensitive to digestion by trypsin after 90 min of chase, and had therefore reached the plasma membrane. In the presence of monensin on the other hand, only 55 % of the haemagglutinin had reached the plasma membrane after 90 min of chase, while after 180 min of chase 94 % had arrived there. Hence, monensin reduces the rate at which haemagglutinin is transported to the plasma membrane. In addition, it should be noted that in the presence of monensin haemagglutinin is not terminally glycosylated, even after 180 min of chase. The internal viral proteins were resistant to digestion, which demonstrates that trypsin did not penetrate the cells.

The presence of the envelope proteins on the surface of untreated MDCK cells at 5 h post-infection was detected by indirect immunofluorescence microscopy (Fig. 5A). The punctate distribution of fluorescence has been shown (Rodriguez Boulan & Pendergast, 1980) to result from the staining of microvilli, which are only on the apical surface of the cells. No staining could be detected when non-immune serum was used. After treatment of the cells with monensin the intensity of fluorescence was much reduced (Fig. 5B), which indicates that this agent greatly reduces the density of envelope proteins in the plasma membrane at 5 h post-infection.

Effect of monensin on viral budding

In untreated monolayers budding of virions began at about 4 h post-infection (Fig.
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6A) and reached a peak at about 7 h post-infection (Fig. 6B). Budding occurred exclusively at the apical surface of the monolayer. In the presence of monensin no budding was detected at 4 h post-infection (Fig. 6C) and even at 7 h post-infection only a few virions are seen (Fig. 6D). This result is consistent with that obtained by

Fig. 4. Effect of monensin on the insertion of haemagglutinin into the plasma membrane of influenza virus-infected MDCK cells. Post-infection maintenance medium contained either no additives (a,b) or 1 μM-monensin (c–f). Cells were labelled 5 h post-infection with [35S]methionine (10 μCi/ml) for 10 min and chased for either 90 min (a–d) or 180 min (e,f) in the presence of either excess unlabelled methionine alone (a,b) or methionine plus 1 μM-monensin (c–f). Cells were then harvested either immediately (a,c,e) or after exposure to trypsin (Sigma; 0.5 mg/ml in PBS) for 15 min at 37°C (b,d,f). The arrows indicate the forms of haemagglutinin produced in the presence of monensin. The fluorograph was scanned using a Joyce-Loebl densitometer scanner (Chromoscan 3).
Fig. 5. Detection of influenza virus envelope proteins in the plasma membrane of infected MDCK cells by indirect immunofluorescence microscopy: the effect of monensin. Post-infection maintenance medium contained either no additives (A) or 1 μM-monensin (B). Confluent monolayers grown on glass coverslips were fixed 5 h post-infection with a 4% (w/v) solution of formaldehyde in PBS (freshly prepared from paraformaldehyde) before exposure to mouse immune serum raised against purified whole virus (10-fold diluted in PBS), followed by FITC-conjugated rabbit anti-mouse IgG (also 10-fold diluted in PBS). Cells were examined by darkground fluorescence using a Leitz Ortholux UV microscope equipped with excitation/suppression filters appropriate to fluorescein. Bar, 10 μm.

immunofluorescence microscopy and with the widely held belief that insertion of the envelope proteins is a prerequisite of budding.
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Fig. 6. Electron micrographs of sections through influenza virus-infected MDCK cell monolayers, showing the effect of monensin on viral budding. A and B. Untreated cells fixed at 4 h and 7 h post-infection, respectively; C and D, cells treated from 1 h post-infection with monensin (1 μM) and again fixed at 4 h and 7 h post-infection, respectively. Bar, 1 μm. Arrows indicate budding virions. Asterisks indicate vacuoles apparently containing virions.

In monensin-treated cells the regular stacks of cisternae that characterize the normal Golgi complex (not shown) were no longer seen. Instead large vacuoles were present, which in some cases (e.g. see Fig. 6c) appeared to contain poorly preserved virions. Budding of virions into Golgi-derived vacuoles after monensin treatment has been observed in other systems (Green et al. 1981) and has been ascribed to an accumulation of envelope proteins in the Golgi complex, which initiates viral assembly here instead of at the plasma membrane, as in untreated cells.

DISCUSSION

The passage through the Golgi complex of proteins en route to the plasma membrane has recently been demonstrated directly by immunoelectron microscopy in the
case of the envelope proteins of Semliki Forest virus (SFV) (Green et al. 1981) and VSV (Bergmann et al. 1981). However, terminal glycosylation, and consequent acquisition of resistance to endo H, can be used as a biochemical indicator of transport through the Golgi complex, since the enzymes involved are known to be localized in this organelle (Dunphy et al. 1981; Griffiths et al. 1982). The time taken for influenza virus haemagglutinin to undergo terminal glycosylation in MDCK cells (approx. 20 min) is comparable to values obtained for other surface glycoproteins in other cell types, such as H-2D\(^k\) histocompatibility antigen in mouse lymphoma cells (Dobberstein, Garoff, Warren & Robinson, 1979), VSV G protein in rat hepatoma cells (Strous & Lodish, 1980) and SFV spike proteins in BHK cells (Green et al. 1981).

The first asparagine-linked oligosaccharide normally found in association with nascent proteins contains three glucose residues and nine mannose residues (Tulsiani, Harris & Touster, 1982). During the early stages of the biosynthesis of a complex oligosaccharide all of the glucose is removed, along with four of the mannose residues, to leave a five-mannose intermediate, which is sensitive to endo H. After the addition of a single N-acetylglucosamine residue this intermediate is then further trimmed to produce a three-mannose oligosaccharide, which is now resistant to endo H (Green et al. 1981). Finally, other sugar residues are added to complete the process of terminal glycosylation. Although they have not yet been characterized, it is likely that the new forms of haemagglutinin found after chasing in the presence of monensin are normally intermediates in this process. The endo H-sensitive form, which is only slightly smaller than HAs, may, for instance, be produced at an early stage in trimming, before the three-mannose oligosaccharide appears, while the smaller endo H-resistant form may be produced after the generation of the three-mannose oligosaccharide but before significant addition of new sugars has occurred.

A 'mixed' effect of monensin has also been reported in SFV-infected chicken embryo fibroblasts (Pesonen & Kääriäinen, 1982). Here the envelope protein E3, which normally has only complex oligosaccharides, was found after monensin treatment in three forms: core glycosylated, terminally glycosylated (but lacking some sialic acid residues) and intermediate. In VSV-infected rat hepatoma cells, on the other hand, the envelope protein G continues to acquire complete resistance to endo H in the presence of monensin (Strous & Lodish, 1980). Hence monensin affects glycoprotein processing differently in different cell types. In the latter system the native secretory glycoprotein transferrin remains sensitive to endo H after monensin, which raises the possibility that these two proteins follow different paths through the cell.

It appears that the sequential processing of asparagine-linked oligosaccharides occurs as the protein migrates through the Golgi complex in a cis to trans direction (Dunphy et al. 1981; Griffiths et al. 1982). The interruption of processing by monensin, therefore, is assumed to represent a block of transport at a particular point within this organelle. It has recently been demonstrated that SFV-infected BHK cells treated with monensin accumulate viral envelope proteins in cisternae in the middle of the Golgi stack (Griffiths, Quinn & Warren, 1983). The binding of nucleocapsids to these cisternae has made it possible to isolate them (Quinn et al. 1983) and to demonstrate that some biochemical functions of the Golgi complex are physically separate. For example
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α1,2-mannosidase and galactosyl transferase have a trans location, while fatty acylation occurs on the cis side of the monensin block. The results obtained in the present study suggest that for influenza virus haemagglutinin in MDCK cells the monensin block is cis to the Golgi elements responsible for the addition at terminal sugars and is also rather poorly defined, in that two processing intermediates are observed.

The use of trypsin to detect haemagglutinin exposed to the extracellular medium has shown that monensin delays rather than inhibits the transport of haemagglutinin to the plasma membrane. In addition, haemagglutinin reaching the plasma membrane in the presence of monensin is not terminally glycosylated. It would appear, therefore, that in monensin-treated cells haemagglutinin may be able to by-pass the trans Golgi cisternae on its way to the plasma membrane. However, further experiments are clearly necessary in order to substantiate this idea.

Monensin had a pronounced effect on the density of envelope proteins at the apical surface of the monolayer, as revealed by immunofluorescence microscopy, and on viral budding. However, these experiments were conducted relatively early in the course of infection and it is impossible to determine from them whether monensin was inhibiting the transport of envelope proteins to the plasma membrane or simply delaying it. Alonso & Compans (1981) concluded from electron micrographs taken 12 h post-infection and from the numbers of virions shed over 24 h post-infection that the budding of influenza virus from the apical surface of MDCK cell monolayers was unaffected by monensin, whereas VSV budding from the basolateral surface was inhibited by more than 90%. These results were taken to indicate that the envelope proteins of the two viruses follow different pathways through the cell and that only one pathway (that taken by VSV G) is sensitive to monensin. The findings presented here regarding the effect of monensin on the processing of influenza haemagglutinin in MDCK cells may have some bearing on this proposal.

Monensin exerts its effects by intercalating into membranes and abolishing ion gradients, through an electroneutral exchange of protons for monovalent cations (Tartakoff, 1980). The observations that the weak base chloroquine can interfere with the targeting by the Golgi complex of newly synthesized lysosomal enzymes to the lysosome in human skin fibroblasts (Hasilik & Neufeld, 1980) and of ACTH to secretory granules in a mouse pituitary cell line (Moore, Gumbiner & Kelly, 1983) indicate that a low intracisternal pH may be essential for normal Golgi function. However, the exchange of intracellular K+ for extracellular Na+ brought about by monensin also appears to be crucial to its disruption of the intracellular transport of proteins (Tartakoff, 1983).

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