Epitope Mapping of the Melanosomal Matrix Protein gp100 (PMEL17)

RAPID PROCESSING IN THE ENDOPLASMIC RETICULUM AND GLYCOsyLATION IN THE EARLY GOLGI COMPARTMENT

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Melanosomes, specific organelles produced only by melanocytes, undergo a unique maturation process that involves their transition form amorphous rounded vesicles to fibrillar ellipsoid organelles, during which they move from the perinuclear to the distal areas of the cells. This depends upon the trafficking and processing of gp100 (also known as Pmel17 and the silver protein), a protein of great interest, because it elicits immune responses in melanoma patients but in which specific function(s) remains elusive. In this study, we have used biochemical and immunochemical approaches to more critically assess the synthesis, processing, glycosylation, and trafficking of gp100. We now report that gp100 is processed and sorted in a manner distinct from other melanosomal proteins (such as tyrosinase, Tyrp1 and Dct) and is predominantly delivered directly to immature melanosomes following its rapid processing in the endoplasmic reticulum and cis-Golgi. Following its arrival, gp100 is cleaved at the amino and at the carboxyl termini in a series of specific steps that result in the reorganization of immature melanosomes to the fibrillar mature melanosomes. Once this structural reorganization occurs, melanogenic enzymes begin to be targeted to the melanosomes, which are then competent to synthesize melanin pigment.

Melanosomes are proving to be rich resources for several major lines of scientific research. First, because they have such distinctive and readily identifiable structural characteristics, they are ideal models for studying mechanisms involved in the biogenesis of subcellular organelles (1–5). Second, because a number of melanosome-specific proteins are localized in those organelles, the processing and trafficking of those proteins via cellular sorting pathways are other fruitful areas of active research (6–8). Third, a number of inherited human pigmentary diseases (9–11) critically assess the synthesis, processing, glycosylation, and trafficking of gp100. We now report that gp100 is processed and sorted in a manner distinct from other melanosomal proteins (such as tyrosinase, Tyrp1 and Dct) and is predominantly delivered directly to immature melanosomes following its rapid processing in the endoplasmic reticulum and cis-Golgi. Following its arrival, gp100 is cleaved at the amino and at the carboxyl termini in a series of specific steps that result in the reorganization of immature melanosomes to the fibrillar mature melanosomes. Once this structural reorganization occurs, melanogenic enzymes begin to be targeted to the melanosomes, which are then competent to synthesize melanin pigment.

To that end, we have now characterized the processing, glyco-
slyation, and trafficking of the melanosomal protein gp100 in human melanocytes. Taken together, the results showed that gp100 is processed and sorted in a manner distinct from other melanosomal proteins and is predominantly delivered directly to Stage I melanosomes following its processing in the ER and cis-Golgi. Following its delivery there, gp100 was cleaved both at the amino and at the carboxyl termini in a series of specific steps that resulted in the transition of Stage I melanosomes to mature Stage II melanosomes, whereupon melanosomal enzymes were delivered to the organelles and pigment was synthesized.

**MATERIALS AND METHODS**

**Cells and Antibodies Used**—MNT-1 pigmented human melanoma cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) as described previously (1). SK-MEL-28 amelanotic human melanoma cells were obtained from the ATCC (Manassas, VA) and were cultured in minimum essential medium (Invitrogen) containing 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2.8 μM sodium bicarbonate, 2 mM l-glutamine, and penicillin/streptomycin.

The monoclonal antibodies HMB45, HMB50, HMB55, and NKI/bet, which recognize different domains of gp100 (22, 23), were purchased from Lab Vision (Fremont, CA). αPEP7h, αPEP1h, and αPEP13h, which are polyvalent antibodies against the carboxyl-terminal regions of human tyrosinase, tyrosinase-related protein 1 (YR1), and gp100, respectively (24, 25). PEP13h is a synthetic peptide (CPIGENSPSSLQQV-CO2H), which corresponds to the carboxyl-terminal sequence of human gp100 (residues 647–661). αSISN is a rabbit polyclonal antibody raised against peptide αSISN (LEGSRNQDWL-GVPR-CO2H), which corresponds to the amino-terminal region of the murine gp100 sequence (residues 24–37) and which cross-reacts with human gp100 because it differs from the mouse sequence by only 1 of the last 10 residues.

Antibodies to the organelle markers Bie, EEA1, and GM130 were purchased from BD Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-rabbit IgG (whole antibody) and horseradish peroxidase-linked anti-mouse IgG were from Amersham Biosciences.

**Electrophoresis and Western Blotting**—Cell extracts were prepared using the M-PER mammalian protein extraction reagent (Pierce), and protein concentrations were measured using the BCA protein assay (Pierce). The cell extracts were mixed with 2× Tris-glycine SDS sample buffer (Invitrogen), supplemented with 0.1% 2-mercaptoethanol, and boiled for 10 min. The samples were then separated using 8% Tris-glycine SDS sample buffer with 0.1% 2-mercaptoethanol by heat-stable radish peroxidase-linked anti-rabbit IgG (whole antibody) and horseradish peroxidase-linked anti-mouse IgG from Amersham Biosciences.

**Glycosidase Digestions**—Endo H and PNGase F (PNGaseF) were purchased from New England Biolabs (Beverly, MA). MNT-1 cell lysates (5 μg) extracted with M-PER were subjected to each glycosidase digestion according to the manufacturer’s instructions. Samples were digested with 1250 units of Endo H or of PNGaseF for 3 h at 37 °C. After the digestion reaction, 1 μg of each cell lysate was subjected to SDS-PAGE, and immunoreactive bands were detected by Western blotting using antibodies as indicated in the legend to Fig. 5.

**RESULTS**

**Expression of gp100 in Pigmented and in Unpigmented Melanoma Cells**—Based on its predicted amino acid sequence, gp100 is initially translated as a native protein of ~70 kDa; it contains a signal sequence at its amino terminus and thus is expected to be immediately taken up into the ER. gp100 has five potential N-glycosylation sites (at positions 81, 106, 111, 321, and 568), and because of its importance as a melanoma-specific antigen, a number of monoclonal antibodies have been described.
generated in mice that recognize it at various epitopes, namely HMB45, HMB50, HMB55, and NKI/beteb (26), although the specific epitopes recognized by those monoclonals have not been adequately defined. As additional probes to study its synthesis and processing, we have produced antibodies in rabbits against peptides that correspond to the amino and carboxyl termini of gp100; these antibodies are termed omSiN (21) and aPEP13h (27), respectively. We were initially interested in characterizing the expression, processing, and subcellular distribution of gp100 in several pigmented and unpigmented human melanoma cell lines and at the same time in mapping the various epitopes of gp100 recognized by those different monoclonal antibodies.

We initially compared the expression profile of gp100 in a pigmented melanoma cell line, MNT-1, and in a number of amelanotic melanoma cell lines, including SK-MEL-28 (Fig. 1). SP1 keratinocytes were used as a negative control. aPEP13h recognized two major specific bands (100 kDa and 26 kDa) in MNT-1 cells, indicating that the carboxyl terminus of gp100 is present in those two bands. aPEP13h also detected a minor band at ~70 kDa. omSiN reacted specifically with the full-length 100-kDa protein band and also with 45-, 30-, 26-, and 10-kDa fragments, which contain the amino terminus of the protein (the 65-kDa fragment is nonspecific because it is also recognized in SP1 keratinocytes). Thus, both aPEP13h and omSiN recognize the full-length 100-kDa form of gp100 in MNT-1 cells, and both antibodies also detect 26-kDa fragments, showing that gp100 is cleaved at a point about 26 kDa from its amino and its carboxyl termini. Interestingly, SK-MEL-28 amelanotic melanoma cells also express gp100, although their processing and PAGE banding patterns are quite distinct from those in MNT-1 cells (e.g. only faint traces of the 26-kDa bands were seen in those cells). Several other amelanotic melanoma cell lines did not express levels of gp100 detectable by Western blot (data not shown).

In contrast, the monoclonal antibody HMB45 reacted only with a low molecular weight heterogeneous fragment of gp100 that has an approximate size of 34–38 kDa. MNT-1 cells reacted positively with HMB45 as did SK-MEL-28 cells at a reduced level, but reactivity with HMB45 was completely negative in SP1 keratinocytes (and in the other amelanotic melanoma cell lines tested). HMB50, HMB55, and NKI/beteb do not work well in the Western blot format, although they function well in immunohistochemistry and in immunoprecipitation formats, as discussed below.

For comparison, we also examined the expression of other melanosomal proteins in those same cell lines. Tyrosinase (Fig. 1), TYRP1, and MART1 (not shown) were strongly positive in the highly pigmented MNT-1 cells, as reported previously (1), and DCT was faintly positive in all melanoma cell lines tested (data not shown). The expression of tyrosinase and MART1 was positive in the amelanotic SK-MEL-28 melanoma cells, but tyrosinase levels in those cells are much reduced and are only partially glycosylated, as recently detailed (28).

To further determine the epitope map and reactivity patterns of the various antibodies to gp100, we performed immunodepletion studies in which extracts of MNT-1 cells were purified over immunopellicity columns containing HMB45, HMB50, HMB55, or NKI/beteb (or IgG as a control). The flow-through (unabsorbed) and the absorbed fractions were then separated by SDS-PAGE and were subsequently reacted in Western blots with aPEP13h (which recognizes the carboxyl terminus of gp100), omSiN, aPEP11h, or control IgG. HMB45 has been reported previously not to work well in immunoprecipitation protocols (22), and that was indeed confirmed in this study, because all gp100 detectable by aPEP13h or by HMB45 was found in the flow-through (unabsorbed) fraction of the HMB45-treated sample and in the control IgG as well (Fig. 2A). gp100 was significantly absorbed by HMB50, HMB55, and NKI/beteb, as shown by the reduced levels of gp100 present in the flow-through fraction unabsorbed by those antibodies. aPEP13h readily detected both the full-length 100-kDa and the 26-kDa fragment of gp100 in samples absorbed by HMB50, HMB55, or NKI/beteb (Fig. 2B), which demonstrates that each of their epitopes is located in the 26-kDa fragment at the carboxy-terminal region of gp100. In contrast, omSiN recognized only the full-length 100-kDa gp100 in samples absorbed by HMB50, HMB55, or NKI/beteb, which is consistent with the data reported above. As controls, no gp100-reactive bands were recognized in extracts reacted with aPEP11h (an antibody that recognizes TYRP1), which was used as a negative control, and reactivity was also negative if no second antibody was used (control).

**Synthesis, Processing, and Stability of gp100**—To assess the synthesis and processing of gp100, we used pulse-chase metabolic labeling of MNT-1 cells and immunoprecipitation with the gp100-specific antibodies (Fig. 3). As an example of the processing of other melanosomal proteins, tyrosinase processing was also examined immediately after the 30-min pulse and at various times of chase up to 24 h. Tyrosinase (detected by aPEP7h) was processed from its native 65-kDa form to the fully...
Figure 2. Immunoabsorption of gp100 by monoclonal antibodies. Extracts of MNT-1 melanoma cells were immunoabsorbed by HMB45 (at 1:1000), HMB50 (at 1:1000), HMB55 (at 1:1000), or NKI/beteb (at 1:1000) or by normal IgG as a control (at 1:1000) as listed across the top of the figure. Proteins that were not absorbed (A) or were immunoabsorbed (B) on those columns were then separated by SDS-PAGE, as detailed for Fig. 1. After transfer to polyvinylidene difluoride membranes, antigens were detected by αPEP13h (at 1:5000), αmSiN (at 1:1250), αPEP1h (at 1:1000), or control IgG (at 1:1000), as noted across the bottom of the figure. Symbols are as defined for Fig. 1. The 26-kDa nonspecific minor band represents the small chain of IgG that elutes from the column in most cases; in some lanes the 26-kDa band derived from gp100 is also seen at that position.

Glycosylation and Sorting of gp100

Tyrosinase is highly resistant to digestion with Endo H as described previously (30) (Fig. 5A). This reflects the fact that tyrosinase is processed through the ER and subsequently the trans-Golgi network, at which point it becomes fully glycosylated and resistant to Endo H (31). Treatment with PNGaseF, which removes all carbohydrate residues, reveals the native protein chain of tyrosinase (~55 kDa).

In contrast, the mature form of gp100 recognized by αPEP13h remains highly sensitive to Endo H treatment, and treatment with PNGaseF has no further effect on its size. These results indicate clearly that gp100 contains N-linked carbohydrate residues and is processed in the ER but that at least the bulk of it is not further glycosylated, thus implying that it is not processed through the trans-Golgi/endoosomal network and must be delivered to melanosomes via another sorting pathway. Although treatment with neuraminidase to remove sialic acid groups has a significant effect on the mobility and heterogeneity of tyrosinase, it has no discernible effect on gp100. Note that the 26-kDa fragment of gp100 is also glycosylated to some extent because it is sensitive to treatment with PNGaseF and lowers the size of that band by about 15 kDa.
Endo H or PNGaseF had no significant effect on the mobility of the 35-kDa fragment of gp100 recognized by HMB45 (except to increase its reactivity with that antibody). A previous report has shown that two mannosidase-specific inhibitors (swainsonine and deoxymannojirimycin) had dramatic effects on the glycosylation of tyrosinase, TYRP1, and DCT but had no detectable effect on the molecular size of gp100 (32). Thus, the sum of these results shows that, although gp100 undergoes early glycosylation events in the ER, it is not further processed through the trans-Golgi/endosomal network.

One would predict that this transition might affect the solubilization characteristics of those epitopes, and we have conducted experiments to examine that using different extraction buffers of increasing solubilization potential (Fig. 5B). As the extraction conditions increase from a nonionic detergent only (Fig. 5B, lanes 1, Triton X-100) to increasing concentrations of an ionic detergent (SDS) and deoxycholate (lanes 2–4) and finally with M-PER (lanes 5), the extraction of the full-length (membrane-bound) gp100 recognized by αPEP7h, whereas gp100 epitopes were detected by αPEP13h and by HMB45.

**Fig. 3.** Pulse-chase metabolic labeling and immunoprecipitation of gp100. MNT-1 melanoma cells were pulse-labeled for 30 min with [35S]methionine and were then chased for various periods of time (hours), as noted above each lane. Solubilized extracts of the cells were immunoprecipitated with antibodies (all at 1:1000), separated by SDS-PAGE as detailed for Fig. 1, and visualized by autoradiography. Symbols are as defined for Fig. 1.

**Fig. 4.** Melanosomal protein stability. MNT-1 melanoma cells were cultured in the presence of cycloheximide for various times as noted, and the treated cells were then solubilized and analyzed by Western blot. Treatment times are noted (in hours) above each lane; tyrosinase was detected by αPEP7h, whereas gp100 epitopes were detected by αPEP13h and by HMB45.
Tyrosinase was detected by αPEP7h, whereas gp100 was detected by αPEP13h and by HMB45. Tyrosinase sorting to early melanosomes is seen clearly in SK-MEL-28 cells, which colocalizes with gp100 to some extent, but the disruption of MNT-1 cells, tyrosinase is delivered to early melanosomes and impaired to some degree in those cells. In the pigmented melanoma cells was then examined using confocal immunohistochemistry (Fig. 6). The yellow color indicates colocalization, whereas distinct signals are red or green. gp100 detected by αPEP13h (Fig. 6, red in all panels) colocalizes strongly with the ER marker (Bip) in MNT-1 (top row) and in SK-MEL-28 (bottom row) melanoma cells and was also found in the early cis-Golgi (GM130). However, very little gp100 colocalized in early endosomes (Fig. 6, EEA1), and in general there was complete separation of those signals, which is consistent with the biochemical and immunochemical studies presented in Figs. 3 and 5. There is also virtually complete segregation of gp100 recognized by αPEP13h with HMB45 (i.e. processed gp100 in Stage II melanosomes), as previously reported in MNT-1 cells (1). Interestingly, however, there was more colocalization of those signals in SK-MEL-28 cells, suggesting that the maturation of melanosomes from Stage I to Stage II is impaired to some degree in those cells. In the pigmented MNT-1 cells, tyrosinase is delivered to early melanosomes and colocalizes with gp100 to some extent, but the disruption of tyrosinase sorting to early melanosomes is seen clearly in SK-MEL-28 cells, and this misrouting results in the amelanotic phenotype of those cells.

**DISCUSSION**

gp100 has been an enigmatic protein with respect to its function in pigmentation, although all studies to date agree on its critical importance as a melanoma marker and as a specific tumor target. In this study, we have shown that gp100 is synthesized as a 70-kDa nascent protein that undergoes immediate early glycosylation in the ER and processing in the cis-Golgi. The bulk of gp100 is then quickly sorted to Stage I early melanosomes without going through the later glycosylation events that are associated with processing in the trans-Golgi. This was demonstrated clearly using pulse-chase metabolic labeling, digestion with glycosidases, and Western immunoblotting. The 100-kDa form of gp100 remains Endo H-sensitive, and its trafficking pathway is quite distinct from the pathway(s) involved in the trafficking of tyrosinase and the other melanosomal proteins (7, 33). A small amount (~5%) of gp100 does seem to undergo additional late glycosylation events that increase its mass to ~110 kDa, a form that is Endo H-resistant (16, 34). The presence of gp100 in the endosomal system has been reported previously as evidence that it is processed through that pathway to reach Stage I melanosomes (6, 16). Such evidence has been obtained primarily using nonmelanocytic cells transfected with wild-type and/or mutant constructs of gp100 and also by confocal immunohistochemistry. Such a model may not be physiologically relevant, because the target organelle (and its melanocyte-specific components) are not expressed in those cells; hence the normal process would be disrupted. The colocalization of gp100 in endosomal compartments does not provide a clue as to whether they are coming or going from melanosomes. In contrast to the conclusions of those previous studies, our results suggest that the gp100 found in the endosomal system is in the process of being removed from the cell.

An interesting consideration is whether other proteins follow a pathway of processing through the Golgi but bypass the endosomal sorting pathway. As one example of such an occurrence, secretory granules do form from the Golgi apart from the endosomal pathway (35), and it is interesting to note that our recent proteomics study (1) shows that early melanosomes do...
**FIG. 6.** Subcellular localization of melanosomal proteins in melanoma cells. MNT-1 (*top row*) and SK-MEL-28 (*bottom row*) melanoma cells were fixed for immunohistochemistry and stained with αPEP13h (at 1:20) and detected by Texas Red (red) as detailed under “Materials and Methods.” The cells were also stained with markers of endoplasmic reticulum (*Bip*, at 1:10), Golgi (*GM130*, at 1:10), early endosomes (*EEA1*, at 1:40), and Stage II melanosomes (*HMB45*, at 1:10) detected by fluorescein isothiocyanate (green). Yellow indicates colocalization of the signals.

**FIG. 7.** Processing and epitope mapping of gp100. gp100 is shown as the complete native protein at the top of the figure and in its processed/cleaved forms in the middle and at the bottom of the figure. Potential glycosylation sites at residues 81, 106, 111, 321, and 568 are indicated as fully glycosylated sites, and Val-467 (V467) refers to the potential cleavage site during the maturation (represented by the large downward arrows) of Stage I to Stage II melanosomes as discussed in the text. The epitopes recognized by the various polyclonal and monoclonal antibodies to gp100 are shown as described under “Results” and previous evidence as discussed in the text. TM, transmembrane domain; [], signal sequence; gray vertical dotted lines, proteolytic cleavage site; inverted Ys, antibody.
in fact contain several proteins thought to be specific to secretory granules. Melanosomes can be considered as secretory granules in some respects, because they are specialized vesicles in which their ultimate purpose is to be secreted from melanocytes. We cannot rule out the possibility that the final delivery of gp100 to Stage I melanosomes occurs from the trans-Golgi, because the sorting and processing machinery to intracellular organelles, including secretory granules, is located there.

Taken together with our recent proteomics analysis of early melanosomes (15), which found a number of ER markers associated with those organelles, and with confocal immunohistochemistry results showing the presence of ER proteins even in late Stage III and IV melanosomes, the biogenesis of early melanosomes as vesicles budding directly from the ER (as proposed more than 4 decades ago) (36) seems to be a reasonable mechanism for their formation. We would extend that proposed pathway to include the fact that gp100 (the existence of which was unknown until just more than a decade ago) is trafficked in those ER/early Golgi vesicles, which become de facto Stage I melanosomes. Small amounts of the other melanosomal proteins (such as TYR and TYRP1) are found in Stage I melanosomes (1, 18), and it now seems likely that they are there by virtue of being trapped in the gp100 vesicles that become Stage I melanosomes, because those other melanosomal proteins are processed normally through the ER and cis- and trans-Golgi. However, the bulk of those other melanosome-specific proteins are delivered via sorting vesicles only following the maturation of Stage I to Stage II melanosomes (1, 8), a process that depends on the subsequent proteolytic cleavage of gp100 at its carboxyl and amino termini and its subsequent incorporation into the fibrillar matrix of melanosomes. The importance of gp100 cleavage to the maturation of early melanosomes is underscored by the fact that melanoma cells (pigmented or amelanotic), wherein gp100 is expressed and cleaved correctly (e.g. MNT-1 cells), do form Stage II melanosomes, whereas melanoma cells that do not express gp100 (or are not able to process gp100 into an HMB45-recognizable form) do not produce Stage II melanosomes and thus do not produce pigment, even if they express tyrosinase and/or the other melanosome-specific proteins.

A number of monoclonal antibodies have been developed over the years that recognize different forms of gp100; Fig. 7 presents a summary of the processing events known to occur for gp100 and the various epitopes recognized by the different gp100 antibodies elucidated in this study. gp100 is synthesized as a 661-amino acid polypeptide, which has an initial mass of 70 kDa. Maresh et al. (37) report that gp100 is glycosylated at three N-linked sites at residues Asn-81, Asn-106, and Asn-111. They further report that gp100 is then cleaved into a small carboxyl-terminal fragment (at residue Val-467) and a larger amino-terminal fragment (37), a scenario confirmed in our study. Our results showed further that a second cleavage occurs, approximately at residues 95–100, which generates a 26-kDa fragment containing the amino-terminal region. PNGaseF digestion of that amino-terminal fragment reveals that ~15 kDa represent carbohydrate residues, the core size of that protein fragment thus being 11 kDa. aPep13h recognizes the carboxyl terminus of gp100 by design, and HMB50, HMB55, and NKI-bet2 recognize epitopes that are nearby based on their recognition of full-length gp100 and also on the fact that the carboxyl terminus fragment cleaved from that protein remains immunoreactive with aPep13h. omSin recognizes the amino terminus of gp100 by design, whereas HMB45 recognizes an internal site on the middle fragment. The sum of those results shows that Asn-321 and Asn-568 are also fully glycosylated, each contributing about 5 kDa to the mass of the protein. The interesting and specific feature of HMB45 is that it recognizes only the processed (cleaved) form of gp100 (hence the 45-kDa surname) and thus is specific for Stage II melanosomes, whereas the other antibodies (monoclonal and polyclonal) recognize intact gp100 in early melanosomes and do not distinguish between stages of melanosomes. At the time of gp100 cleavage, the carboxyl-terminal epitope recognized by aPep13h remains in the melanosomal membrane (1, 16), but reactivity with that antibody is then quickly lost, suggesting that it is further degraded soon thereafter. Reactivity with HMB45 is itself lost as melanin is produced and deposited on the melanosomal matrix presumably by physical masking of the gp100 epitope by that pigment. The conformation-dependent nature of the epitope recognized by HMB45 allows us to confirm that the gp100 cleavage events seen using biochemical analyses actually occur in vivo.

An interesting and important question therefore is which protease(s) is involved in the processing of gp100 to its truncated form (steps that are critical to the maturation of melanosomes)? It will be important for future study to identify the protease(s) involved in gp100 trimming and the sites of those proteolytic cleavage events that play such important roles in determining melanosome function and pigmentation and in the generation of gp100 epitopes recognized by the immune system in human melanoma cells. Recently, the involvement of pro-protein convertases in the initial cleavage and processing of gp100 near its carboxyl terminus in a post-Golgi compartment was reported (20), and the presence of a related enzyme, pro-hormone convertase, has also been reported in melanosomes (38), as have a number of different peptidases and proteases (15). Future study will naturally be directed toward identifying those proteolytic components apparently present in early melanosomes, and clues should become available as our proteomics analysis of Stage I melanosomes proceeds.

The importance of gp100 processing goes beyond its implications for melanosome biogenesis and maturation, because it plays a role in the generation of gp100 peptides and their presentation on the surface of melanoma cells, an important criterion to the recognition of those tumor cells by the host immune system.

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