Functional Interactions between the Fusion Protein and Hemagglutinin-Neuraminidase of Human Parainfluenza Viruses

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The fusion glycoprotein (F) and hemagglutinin-neuraminidase (HN) genes of human parainfluenza virus type 2 (PI2) were molecularly cloned and expressed in HeLa-T4 cells by using the vaccinia virus-T7 transient expression system. Expression of the F and HN proteins was detected by using immunoprecipitation and surface immunofluorescence staining. Although the F protein was found to be cleaved into F₁ and F₂ and expressed on cell surfaces, no cell fusion was observed. However, cotransfection of the F-protein gene together with the PI2 HN gene resulted in significant levels of cell fusion. Cell fusion was also observed when separate cell cultures were transfected with the HN and F genes and the F-expressing cells were mixed with the HN-expressing cells. Surprisingly, when the PI2 F protein was expressed together with the parainfluenza virus type 3 (PI3) HN protein, no fusion was detectable in the transfected cells. Similarly, no fusion was found upon coexpression of the PI2 HN and PI3 F proteins. However, coexpression of the PI3 F and HN proteins resulted in extensive cell fusion, which resembled the PI2 coexpression result. These results indicate that under the conditions used, the F protein is unable to cause fusion by itself and the HN protein provides a specific function in cell fusion which cannot be provided by another paramyxovirus attachment protein. Further, the results suggest that a type-specific functional interaction between the F and HN proteins is involved in mediating cell fusion.

The two membrane glycoproteins of paramyxoviruses, the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins, form spikelike projections on the outer surface of the viral envelope. The HN protein possesses receptor-binding and neuraminidase activities, whereas the F protein is directly involved in virus-induced membrane fusion which is required for the penetration of the virus (2, 26). The fusion proteins of paramyxoviruses are synthesized as a precursor, F₀, which is subsequently cleaved by a host cell protease into the biologically active form of the fusion protein, composed of F₁ and F₂ subunits linked by disulfide bonds (8, 27). An extremely hydrophobic domain is exposed after cleavage at the NH₂ terminus of the F₁ subunit, which probably undergoes a conformational change (7, 10). This hydrophobic domain is highly conserved among paramyxovirus F proteins and is considered likely to be directly involved in mediating membrane fusion (5).

Several groups have investigated the induction of cell fusion by expression of the paramyxovirus F proteins and the role of the HN protein in the fusion process. Studies with simian virus 5 and measles virus indicated that expression of the F protein alone resulted in cell fusion (1, 21, 22). However, other studies with measles virus (31), bovine (25) as well as human (3, 17) parainfluenza virus type 3 (PI3), and Newcastle disease virus (16) indicated that the induction of cell fusion required both the F and HN proteins. At this point, it is not certain whether these differences reflect intrinsic differences in the fusion activity of various F proteins or whether the use of different host cell systems or vector systems may contribute to the results observed.

The present studies were undertaken to further define the requirements for cell fusion induced by human parainfluenza viruses. We investigated the expression of the F and HN proteins of human parainfluenza virus type 2 (PI2) by using

the vaccinia virus-T7 transient expression system. To investigate the requirements for cell fusion mediated by the F protein, we compared the results of expression of F alone with coexpression of homologous or heterologous HN proteins or other viral attachment proteins.

MATERIALS AND METHODS

Viruses and cells. PI2 (Greer strain) was obtained from the Division of Research and Resources, National Institutes of Health, Bethesda, Md. The virus was adapted to grow on LLC-MK₂ cells and purified as described previously (11). The vaccinia virus-T7 recombinant virus (WR strain) was obtained from Bernard Moss. HeLa-T4, baby hamster kidney (BHK-21), and HEP-2 cell lines were purchased from the American Type Culture Collection, Rockville, Md. Cells were grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum.

Construction of recombinant plasmids. (i) **PI2 F clone (pGFsn).** Two overlapping partial cDNA clones (A5 and E4) of the PI2 F gene (11) inserted in pGEM-3 plasmids (Promega Biotec, Madison, Wis.) at the *Sma*I site were cut with *Pst*I, the resulting DNA fragments were eluted from the agarose gel, and a full-length PI2 F gene (pGF) containing 180 nucleotides in the 5'-noncoding region was obtained by religating the DNA fragments with T4 DNA ligase. To obtain a cDNA clone with a shorter 5'-noncoding sequence (pGFsn), pGF was cut with *Hae*III and *Sal*I, and a clone containing only 15 nucleotides in the 5'-noncoding region was eluted from the agarose gel and then ligated with pGEM-3 cut with *Sma*I and *Sal*I.

(ii) **PI2 HN clone.** Poly(A)⁺ RNA from PI2-infected LLC-MK₂ cells was isolated and enriched by two cycles of oligo(dT)-cellulose column chromatography and used as a template for cDNA synthesis (11). cDNA encoding the HN gene was synthesized by using a specific synthetic oligonucleotide primer complementary to the 3' end of the mRNA

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sequence (12) and a commercial cDNA synthesis kit (Amersham Corp., Arlington, Ill.). The cDNA was inserted in the *Sma*I site of pGEM-3Zf(-) (Promega) and transformed into *Escherichia coli* DH5 α competent bacteria (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and colonies were screened by colony hybridization with a specific synthetic oligonucleotide probe corresponding to nucleotides 868 to 887 of the published HN sequence (12).

The PI3 F and HN cDNA clones were gifts from Mark Galinski. The two genes were subcloned into pGEM-3. The human immunodeficiency virus type 2 (HIV-2) ST *env*, simian immunodeficiency virus (SIV)mac239 *env*, and HIV-2 ROD *env* clones were obtained from Mark J. Mulligan (18).

Infection and transfection. Cells were grown in 35-mm dishes at 37°C in Dulbecco's modified Eagle's medium containing 10% heat-inactivated newborn calf serum. Infection of cells with wild-type PI2 was previously described (11). For expression studies, confluent monolayers of cells were infected with the vaccinia virus-T7 recombinant (4) at a multiplicity of infection of 10, incubated at 37°C for 1 h, and then washed with warm phosphate-buffered saline (PBS). Cells were then transfected with 5 μ g of plasmid DNA with lipofectin (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) in 0.5 ml of Dulbecco's modified Eagle's medium. Dulbecco's medium with 5% newborn calf serum was added after cells were incubated with the transfection mixture at 37°C for 5 h. After a 16- to 20-h incubation, cells were analyzed by immunoprecipitation or immunofluorescence assays.

Rabbit antisera against PI2. Purified PI2 was emulsified with Freund's complete adjuvant and injected intramuscularly into the hind legs of rabbits. Rabbits were boosted intramuscularly at weekly intervals for 3 weeks with emulsified viral proteins in Freund's incomplete adjuvant. A single intravenous dose of virus without adjuvant was injected 5 days before bleeding. Each rabbit received a total of 250 μ g (five times 50 μ g) of viral protein. Serum prepared from clotted blood was stored at -70°C until used.

Radiolabeling and immunoprecipitation. Transfected, infected, or mock-infected cells were starved in Dulbecco's modified Eagle's medium lacking methionine and cysteine for 15 min and labeled with 60 μ Ci of [³⁵S]methionine per 35-mm dish for 1 h at 37°C. Immunoprecipitation was performed as described previously (24), and the immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Surface immunofluorescence staining. Indirect immunofluorescence was performed as described by Paterson et al. (22). In general, cells were grown on glass coverslips in 24-well plates and transfected with plasmid DNA or infected with wild-type PI2 as described above. After transfection or infection, cells were washed with ice-cold PBS three times before being fixed with 1% paraformaldehyde in PBS. Rabbit anti-PI2 antibody (1:20 dilution in PBS) was added onto cell monolayers, and cells were then incubated at 4°C for 20 min. Cells were washed with ice-cold PBS three times, and then goat anti-rabbit immunoglobulin G fluorescein-conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was added and was then incubated at 4°C for 20 min. Cell surface fluorescence was examined by fluorescence microscopy with a Nikon Optiphot microscope.

Quantitation of cell fusion. Cell fusion was measured by polykaryon formation and expressed as (number of nuclei in polykaryons/number of total nuclei) $\times$ 100. Cells were counted in five different fields under a light microscope.

FIG. 1. Immunoprecipitation of PI2 proteins expressed by using the vaccinia virus-T7 system. HeLa-T4 cells transfected with plasmids containing the PI2 F or HN gene or infected with wild-type PI2 were radiolabeled with [³⁵S]methionine (A) at 37°C for 1 h or with [³H]glucosamine (B) for 6 h. The cells were lysed and extracted in RIPA buffer. The PI2 proteins were immunoprecipitated with rabbit anti-PI2 polyclonal antibody and analyzed on SDS-polyacrylamide gels by electrophoresis. Lanes 1, wild-type PI2-infected cell lysates; lanes 2, PI2 F-transfected cell lysates; lanes 3, PI2 HN-transfected cell lysates. Cell lysates were electrophoresed on SDS-10% polyacrylamide gels in the presence of the reducing agent 2-mercaptoethanol.

RESULTS

Expression of the PI2 F and HN proteins. To analyze the expression of the PI2 F and HN proteins, we used the vaccinia virus-T7 transient expression system (4). HeLa-T4 cells, which are relatively resistant to the cytopathic effect of vaccinia virus (13), were infected with the vaccinia virus-T7 recombinant and transfected with recombinant plasmids containing the PI2 F or HN gene. The expression of the PI2 F protein was detected by immunoprecipitation of transfected cell lysates radiolabeled with [³⁵S]methionine by using rabbit anti-PI2 polyclonal antibody (Fig. 1A). Under reducing conditions, a fraction of the F proteins in both PI2 F-transfected (lane 2) or PI2 virus-infected (lane 1) cell lysates was found to be cleaved, and the F₁ subunit of the fusion protein was detectable, although the F₂ subunit was not resolved. However, when cells were labeled with [³H]glucosamine (Fig. 1B) and analyzed in the presence of reducing agent, both the F₁ and F₂ subunits of the F protein were resolved. The HN proteins appeared to be present as both monomeric and oligomeric forms under nonreducing conditions (data not shown). The slower migration of the expressed PI2 F protein compared with that of the F protein of wild-type PI2 may be a result of removal of sialic acid from the F protein by the neuraminidase activity of HN. This is more obvious on a nonreducing gel (data not shown), because the unreduced F₁-F₂ complex is more highly glycosylated than the F₁ subunit (11). These results indicated that the PI2 F and HN genes were expressed efficiently and that a fraction of the F proteins was cleaved into F₁ and F₂ subunits in HeLa-T4 cells.

The surface expression of the PI2 F and HN proteins was examined 20 h posttransfection by indirect immunofluorescence staining of paraformaldehyde-fixed cells, using rabbit anti-PI2 antiserum (Fig. 2). PI2 F-transfected cells (Fig. 2A)

FIG. 2. Indirect immunofluorescence staining of the F and HN proteins expressed at the cell surface. HeLa-T4 cells transfected with recombinant plasmids or infected with PI2 were washed with PBS, fixed in 1% paraformaldehyde, and sequentially incubated at 4°C with rabbit antiserum against PI2 and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. (A) PI2 F-transfected cells. (B) PI2 HN-transfected cells. (C) Wild-type PI2-infected cells. (D) Vaccinia virus-T7-infected cells.

and HN-transfected cells (Fig. 2B) showed levels of surface fluorescence similar to those found in wild-type virus-infected cells (Fig. 2C). No surface fluorescence was observed on vaccinia virus T7-infected control cells (Fig. 2D) or mock-infected cells (not shown). These results indicate that the PI2 F and HN proteins expressed in HeLa-T4 cells are efficiently transported to the cell surface.

Induction of cell fusion. Although the PI2 F protein was cleaved and detectable on the surfaces of HeLa-T4 cells, no cell fusion was observed (Fig. 3A). No evidence for cell fusion was seen, even though various concentrations of the plasmid DNA were used for transfection and transfected cells were examined for cell fusion for up to 36 h, until most of the cells showed a cytopathic effect caused by the vaccinia virus-T7 infection. In contrast, infection by wild-type PI2 resulted in cell fusion in HeLa-T4 cells after 16 to 20 h of incubation at 37°C (Fig. 3B). Trypsin treatment was also used to investigate whether cell fusion might be detected if cleavage of the F protein is enhanced. However, syncytium formation was not observed in experiments in which amounts of trypsin varying from 0.1 to 25 µg/ml were added to the culture medium (data not shown).

As described above, several previous reports indicated that the expression of both the HN and F proteins was required for cell fusion. To investigate the effect of coexpression of the two proteins, we cotransfected HeLa-T4 cells with the PI2 F and HN genes. Extensive cell fusion was observed in the cotransfected cells by 8 to 10 h posttransfection (Fig. 3C), whereas no effect was observed in cells transfected with HN alone (Fig. 3D) or in vaccinia virus-T7-infected cells (Fig. 3E). By surface immunofluorescence staining, it was observed that HeLa-T4 cells cotransfected with the PI2 F and HN genes showed fluorescence on the surfaces of large syncytia (Fig. 3F). These results indicate that under the conditions used, the F protein alone was unable to cause cell fusion, and the presence of both the F and HN proteins was necessary for syncytium formation to occur.

Cell fusion requires expression of F and HN proteins from the same virus serotype. If the function provided by the PI2 HN protein is to facilitate cell-cell attachment, it would be expected that this function could also be supplied by the HN protein of a different paramyxovirus type. To examine this possibility, we determined whether the HN protein of PI3 could replace the PI2 HN protein in facilitating cell fusion. Surprisingly, when PI2 F was coexpressed with PI3 HN, no membrane fusion was observed in transfected cells (Fig. 4A). Similarly, coexpression of PI2 HN and PI3 F resulted in no fusion (Fig. 4B). However, coexpression of the PI3 F and HN proteins resulted in extensive cell fusion (Fig. 4D), which resembled the PI2 F and HN coexpression result (Fig. 4C). These data indicate that at least for PI2 and PI3, only the coexpression of glycoproteins of the same virus types results in cell fusion, suggesting that a type-specific interaction is required for these two glycoproteins to induce syncytium formation. We also determined whether a heterologous F or HN protein had an inhibitory effect on cell fusion when coexpressed with the PI2 F plus HN proteins. We observed that when either the PI3 HN or PI3 F gene was cotransfected together with the PI2 F and HN protein genes, the extent of fusion was similar to that observed with the PI2 genes alone (data not shown). Thus, the presence of heterologous F or HN protein does not inhibit cell fusion.

The envelope glycoproteins of some lentivirus isolates are defective in syncytium formation, but it is not known whether their reduced fusogenicity is due to a defect in the attachment function or the fusion activity per se (18, 19). We investigated whether the PI2 F or HN protein can complement the fusion activity of these retrovirus *env* proteins and hence reveal which part of the *env* protein is defective. The PI2 F or HN gene was therefore cotransfected in HeLa-T4 cells with *env* genes of two nonfusogenic retroviruses, HIV-2 ST or SIVmac239 (18, 19). However, no cell fusion was observed, whereas cells transfected with the fusogenic HIV-2 ROD *env* gene alone showed extensive membrane fusion (data not shown). These results indicate that the PI2 F

FIG. 3. Induction of fusion in transfected HeLa-T4 cells. Cells transfected with recombinant plasmids or infected with PI2 were incubated at 37°C for 12 to 16 h. (A) Cells expressing PI2 F. No fusion was observed throughout the entire monolayer. (B) Cells infected with wild-type PI2. (C) Cells cotransfected with PI2 F and HN genes. About 65% of cells were found to become giant cells when 5 μ g of plasmid DNA was used for transfection. (D) Cells transfected with PI2 HN. (E) Cells infected with vaccinia virus-T7. (F) Indirect immunofluorescence staining of HeLa-T4 cells expressing PI2 F and HN.

or HN protein was unable to complement the defect in fusion activity of these retrovirus *env* proteins.

To confirm the requirement for glycoprotein type specificity in cell fusion, we performed a double-blind coexpression experiment by transfection of F and HN of PI2, or PI3, or retrovirus *env* genes in various combinations (Table 1). Of nine combinations used for cotransfection, only coexpression of PI2 F with PI2 HN or PI3 F with PI3 HN showed cell fusion, which was found to occur at high levels.

Hsu et al. (9) reported that in the presence of wheat germ agglutinin (WGA), reconstituted vesicles containing the F protein of Sendai virus induced membrane fusion. They proposed that in the intact virus the attachment function is provided by the HN protein and that WGA could substitute for this function, inducing cell fusion. To investigate whether

the PI2 F protein can mediate cell fusion in the presence of this lectin, we added WGA to the medium at a concentration of 10 μ g/ml after cells transfected with PI2 F were incubated at 37°C for 14 h. During a 24-h incubation at 37°C, no membrane fusion was observed (data not shown). Similar results were obtained with the PI3 F protein. This result indicates that in the present system, the function provided by the HN protein in cell fusion cannot be replaced by WGA.

Expression of F and HN in separate cells. To determine whether cell fusion requires the expression of both F and HN in the same cell, we transfected separate cell cultures with the F and HN genes and mixed the resulting cells. Cell fusion was observed when F-transfected cells were suspended and mixed with HN-transfected cells (Fig. 5). Cell

FIG. 4. Type specificity of envelope glycoprotein interaction. HeLa-T4 cells cotransfected with the homologous or heterologous F and HN protein genes were examined for cell fusion after 16 h of incubation at 37°C. (A) Cells cotransfected with PI2 F and PI3 HN genes. (B) Cells cotransfected with PI2 HN and PI3 F genes. (C) Cells cotransfected with PI2 F and PI2 HN genes. (D) Cells cotransfected with PI3 F and PI3 HN genes. Syncytium formation was observed only in cells transfected with homologous HN and F gene pairs, not with heterologous combinations of genes.

fusion resulted from mixing of cells in any of three ways: HN-transfected cells were suspended with 50 mM EDTA and added onto F-transfected monolayers and incubated at 37°C overnight, or vice versa (data not shown), or the F- and HN-transfected cells were separately suspended and mixed. Fusion was observed after 8 to 10 h of incubation following cell mixing. Cell fusion was not observed in these experiments when longer EDTA treatment or vigorous washing was employed, which was found to cause the suspended cells to lose viability as measured by trypan blue staining. When cells expressing the HN and F proteins from different serotypes were mixed, no cell fusion occurred. These results suggest that the F and HN glycoproteins on different cells can interact to induce cell fusion.

Cell fusion in various cell types. To examine whether the requirements for cell fusion were similar in other cell types, HEp-2 and BHK cells were transfected with the F or HN

gene or cotransfected with the two protein genes. Similar to the results observed with HeLa-T4 cells, transfection with F alone resulted in no fusion, whereas cotransfection with PI2 F and HN or PI3 F and HN induced cell fusion (data not shown). The extent of cell fusion in HEp-2 cells was less than that in HeLa-T4 cells when using the same amount of DNA transfected with lipofectin. As with HeLa-T4 cells, no cell fusion was found upon coexpression of heterologous HN and F glycoproteins. These results further support the conclusion that a type-specific interaction occurs between the two parainfluenza virus membrane glycoproteins and is required for induction of cell fusion in various cell types.

DISCUSSION

We investigated the expression and cell fusion activity of parainfluenza virus glycoproteins in several cell types, using the vaccinia virus-T7 polymerase transient expression system. Although the PI2 F protein was expressed at high levels, cleaved, and transported to the cell surface, no cell fusion was detectable. However, extensive cell fusion was observed in cells cotransfected with the PI2 F and HN glycoproteins. Thus, the results indicate that both the F and HN proteins are necessary for syncytium formation in the present system. We obtained similar results in several cell types, all of which exhibited a requirement for expression of both HN and F in order for fusion to occur. Several other lines of evidence have also been obtained previously to support the conclusion that the HN protein plays a role in the fusion process with other paramyxoviruses (14, 15, 20,

TABLE 1. Double-blind assay for cell fusion^a

DNA	% of nuclei			
	PI2 F	PI3 F	Simian virus 5 F	HIV-2 ST <i>env</i>
PI2 HN	65	<1	<1	<1
PI3 HN	<1	70	<1	<1
SIVmac239 <i>env</i>	<1	<1	<1	<1

^a Five micrograms of DNA of each clone was used to transfect HeLa-T4 cells. Cell fusion was examined 8 to 36 h posttransfection. Percentages refer to nuclei involved in polykaryon formation.

FIG. 5. Fusion of cocultivated cells expressing the F or HN protein. Separate cultures of HeLa-T4 cells were transfected with the F and HN genes and incubated at 37°C for 16 h. Cells were washed with warm PBS and suspended by treatment with 50 mM EDTA at room temperature for 2 to 3 min. After being washed three times with warm PBS, cells expressing the PI2 HN protein were added onto the monolayer of the PI2 F-expressing cells (A) or the PI2 F- or HN-expressing cells were both suspended and mixed (B). Mixed cells were then incubated at 37°C for 8 to 12 h before cell fusion was examined by light microscopy. (C) Mixing of PI2 F- with PI3 HN-expressing cells. (D) Mixing of PI3 F- with PI2 HN-expressing cells. Cell fusion was observed only after mixing of homologous protein-expressing cells.

23, 28–30). Recently, Moscona and Peluso (17) reported that cells persistently infected with PI3 were completely resistant to fusion with each other but readily fused with uninfected cells. They demonstrated that the inability of these cells to fuse with each other was due to a lack of cell surface neuraminic acid and that the HN protein function was necessary for cell fusion. It was proposed that the interaction of the HN protein with neuraminic acid is involved in membrane fusion but that fusion is mediated by an interaction more complex than mere juxtaposition of the cell membranes.

Hsu et al. (9) previously reported that the attachment function for membrane fusion provided by the HN protein, which presumably brings the F protein and the target cells into close proximity, could be provided by other binding agents such as WGA. In the present study, neither other viral attachment proteins which were coexpressed with the F protein in the same cell nor the addition of WGA could replace the homologous HN protein function. The finding that WGA could not substitute for the HN protein in induction of cell fusion is consistent with results reported for cells persistently infected with PI3 (17). Henis et al. (6) have reported a correlation between cell-cell fusion and the lateral mobility of the viral glycoproteins in the target cell membrane. They demonstrated that immobilization of the viral glycoproteins on cell surfaces by cross-linking with concanavalin A resulted in inhibition of cell-cell fusion, but such treatment was much less effective in inhibiting virus-cell fusion. This may be related to the observation that lectins

including WGA were reported to facilitate F-mediated fusion of intact virions or reconstituted viral envelopes, but we observed no effect on cell fusion in the present study. Henis et al. (6) also reported that immobilization of either F or HN by cross-linking with antibodies eliminated their ability to induce cell-cell fusion, suggesting that the HN protein has a role in the induction of cell fusion.

A surprising result was obtained when we mixed F-expressing cells with HN-expressing cells: the separately transfected cells also fused under these conditions. These results suggest that fusion occurs even though the glycoproteins are being expressed on membranes of different cells. This result is different from those reported by Morrison et al. (16), who found that mixing of Newcastle disease virus F-expressing cells with HN-expressing cells did not result in cell fusion, whereas cell fusion occurred when the two glycoproteins were expressed in the same cell. The differences in these observations could depend on the expression levels of the F and HN proteins or on other differences existing in the cell types or vector systems. We also cannot exclude the possibility that transfer of glycoproteins or plasmids between cells occurred in these experiments; however, such a transfer would need to be very efficient in that most cells in the culture were found to undergo fusion.

The present finding that the F protein can induce cell fusion only in the presence of the homologous HN protein suggests that a type-specific interaction between the two membrane glycoproteins is crucial for the induction of cell fusion. It is possible that the two proteins could specifically

contact each other to form a complex which results in fusion activity. Also, some other previously unidentified activity of the HN protein may play a role in modifying the F protein or the target cell. Since expression of a heterologous PI3 protein did not block fusion induced by PI2 F plus HN, it seems unlikely that the heterologous proteins form a nonfunctional complex. The binding and attachment of the HN protein to neuraminic acid is presumably involved in the membrane fusion process, but it appears to be insufficient for fusion to take place, since the HN proteins are capable of binding to sialic acid on cell surfaces irrespective of the virus serotype. It will be of interest to identify the precise domains of the F and HN proteins involved in their functional interaction, as well as their physical relationship on cell surfaces.

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