H^+-induced Membrane Insertion of Influenza Virus Hemagglutinin Involves the HA2 Amino-terminal Fusion Peptide but Not the Coiled Coil Region*

(Received for publication, December 26, 1995, and in revised form, March 14, 1996)

Peter Durrer, Carmela Galli, Stefan Hoenke, Chantal Corti, Reinhard Glück, Thomas Vorherr, and Josef Brunner†

From the Laboratory of Biochemistry, Eidgenössische Technische Hochschule Zürich (ETHZ), ETH-Zentrum, CH-8092 Zürich, Switzerland, the Swiss Serum and Vaccine Institute, P. O. Box 2707, CH-3021 Berne, Switzerland, and the Hoffmann-La Roche A.G., Grenzacherstrasse 124, CH-4002 Basel, Switzerland

Fusion of influenza virus with target membranes is induced by acid and involves complex changes in the viral envelope protein hemagglutinin (HA). In a first, kinetically distinct step, the HA polypeptide chain 2 (HA2) is inserted into the target membrane bilayer. Using hydrophobic photo-labeling with the phospholipid analogue 1-O-hexadecanoyl-2-O-[9-[[2-[125I]iodo-4-(trifluoromethyl-3-diazirin-3-yl)benzyl]oxy]carbonyl-Jnonanoyl]-sn-glycero-3-phosphocholine, we identified the segment that interacts with the mem-
brane. The sole part of the HA2 ectodomain that was labeled with the membrane-restricted reagent is the NH2-terminal fusion peptide residues 1-22. No labeling occurred within the long coiled coil region generated during the acid-induced conformational transition (Bul-
lough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Nature 371, 37-43). These data strongly suggest that the coiled coil region of HA2 does not insert into the lipid bilayer. This conclusion is in variance with the recent suggestion (Yu, Y. G., King, D. S., and Shin, Y.-K. (1994) Science 266, 274-276) that the coiled coil of HA may splay apart and insert into the target membrane, providing a mechanism by which the viral and the target membrane may come in close apposition.

Enveloped viruses enter cells by a process that involves fusion of the viral envelope with a membrane of the host cell (for recent reviews, see Refs. 1-5). Fusion is catalyzed by viral envelope glycoproteins, among which hemagglutinin (HA), 1 the fusion protein of influenza virus, is best characterized and has provided the paradigm for viral fusion proteins. Following binding of influenza virus to sialylated surface receptors, a step also mediated by HA, the virus is internalized. In the acidic environment of the endosomal compartment, the HA protein switch from an inactive to a fusion-active state, promoting fusion of the viral envelope with the endosomal membrane.

Fifteen years ago, the atomic structure of BHA, the brome-
main-solubilized form of HA, has been determined (6). Perhaps the most amazing aspect of this structure is that the HA2 NH2-terminal region, the so-called fusion peptide, is buried inside the homotrimeric HA globular structure. At the pH of fusion this peptide becomes exposed and can insert into the target membrane bilayer, as suggested by photolabeling studies (7, 8). How this may happen can be envisioned from the pH 5 structure of TBHA2, a proteolytic fragment lacking most of HA1 as well as the fusion peptide itself (9). A 28-residue loop region that connects a short helix to a long helical stem is recruited to extend the tripeptide strands of the helical coiled coil toward the amino terminus of HA2. In this way the fusion peptide moves by at least 100 Å to one tip of the molecule and can project toward the target membrane above the globular head domains.

However, this structural reorganization does not explain how the two membranes are brought together. On the basis of their finding, that synthetic 40-residue peptides corresponding to the loop regions of native HA inserts into model lipid mem-

branes at the pH of fusion, Yu et al. (10) proposed that, after initial attachment of the fusion peptide the coiled coil splays apart and inserts into the lipid bilayer of the target membrane, providing the mechanism for membrane merging.

In extending previous investigations (7, 8, 11-14), we now identify the polypeptide segment within HA2 that inserts into the membrane bilayer. Using the photoreactive lipid 125I-TID-

PC/16 (15) as the labeling reagent and new protocols for HA2 fragmentation, we show that the sole part of HA that becomes labeled in a pH-dependent manner is the NH2-terminal fusion peptide. The same is the case for BHA, the soluble form of HA, which inserts into membranes solely through its NH2-terminal fusion peptide. In contrast, synthetic loop-40 peptide in the presence of liposomes bearing 125I-TID-PC/16, becomes labeled in a pH-dependent manner.

MATERIALS AND METHODS

Chemicals and Reagents—Egg PC (grade I) was from Lipid Products (South Nutfield, United Kingdom); POPC from Bachem Feinchemi-
kalien AG (Bubendorf, Switzerland) and POPG from Avanti Polar Lip-
ids, Inc. (Birmingham, AL). 125I-TID-PC/16 and 125I-TID-BE were pre-
pared from the corresponding tin precursors as described (15, 16). The product was stored as a solution in toluene/ethanol (2:1, v/v). Thermo-
lysis was from Sigma and BNPS-skatole from Fluka (Buchs, Switzerland). Prior to use, BNPS-skatole was crystallized from acetone.

Viruses—The X-31 recombinant strain of influenza virus was propa-
gated in the allantoic cavity of embryonated eggs and purified as
described before (17). The lipid/protein ratio (micromoles of phospho-
lipid per mg of protein) of the viruses was 0.22. The viruses were
dispersed in fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM
MES, and 5 mM HEPES, pH 7.3) and kept at 4 °C.

Preparation of LUVs—LUVs were prepared by the extrusion tech-
nique of Hope et al. (18) using polycarbonate filters with a 0.2-μm pore
size.

Photolabeling—The Eppendorf tube containing the liquid sample
was placed in a Pyrex glass vessel mounted approximately 10 cm from
a SUSS LH 1000 lamp-house (KARL SUSS, Waterbury Center, VT)
equipped with an Osram HBO 350 short-watt high pressure mer-
cury lamp. Photolysis was performed for 30 s at a light intensity of 30
milliwatts cm⁻². Under these conditions more than 90% of the reagent
is photolyzed.

Isolation of 125I-TID-PC/16-labeled HA Polypeptides—To the pho-
tolabeled viruses (0.5 ml) were added 3 volumes of chloroform/methanol
(1:2, v/v). After 1 h at room temperature, the precipitated protein was
collected by sedimentation (10 min at 14,000 rpm in an Eppendorf
centrifuge). The protein was dissolved in sample buffer (95 °C for 3 min)
and subjected to SDS-PAGE (nonreducing conditions) using a Tris/
Tricine-buffered system (19). The HA band, visualized by brief staining
with Coomassie Brilliant Blue R-250, was excised and the protein
electroeluted at 50 mA for 4 h using a homemade apparatus. The elution
buffer contained Tris (25 mM), glycine (0.18 M) and 0.1% SDS. The
protein solution (1–2 ml) was concentrated by a Centricon 30
microconcentrator (Amicon) and re-electrophoresed under reducing
conditions. After staining, the HA1 and HA2 bands were excised and
the gel slice containing HA1 was processed for protein quantification
using the Coomassie Brilliant Blue R-250 staining/extraction procedure
of Ball (20). Stain was compared with that of reference samples deter-
mind by a modified Lowry procedure (21) with bovine serum albumin
as a standard. HA2 was electroeluted, concentrated in Centricon 10
microconcentrator, precipitated with 9 volumes of acetone (this step
served to remove the Coomassie blue stain), and dried in vacuo.

BNPS-Skatole Cleavage of (B)HA2 and Separation of Fragments—
HA2 or BHA2 was electroeluted from the SDS-polyacrylamide gel and
the protein solution was concentrated using a Centricon 10 micro-
concentrator (Amicon) and re-electrophoresed under reducing condi-
tions. After staining, the HA1 and HA2 bands were excised and the
gel slice containing HA1 was processed for protein quantification
using the Coomassie Brilliant Blue R-250 staining/extraction procedure
of Ball (20). Stain was compared with that of reference samples deter-
mind by a modified Lowry procedure (21) with bovine serum albumin
as a standard. HA2 was electroeluted, concentrated in Centricon 10
microconcentrator, precipitated with 9 volumes of acetone (this step
served to remove the Coomassie blue stain), and dried in vacuo.

Thermolysin Cleavage of 125I-TID-PC/16-labeled BHA—To the solu-
tion of photolabeled BHA (30 μg of protein in 136 μl of fusion buffer adjunc-
tional SDS) was removed by ion pair extraction as described previously (22). BNPS-skatole cleavage was performed in 60% acetic acid (0.5 μg of protein/μl) in the presence of 1 part of tyrosine
and 10 parts of BNPS-skatole (by weight) under nitrogen for 20 h in the
dark. Subsequently, excess cleavage reagent and by-products were ex-
tacted with 3 volumes of ethyl acetate. The aqueous phase was added to the Speed Vac, and the residue dissolved in sample buffer for subse-
quent SDS-PAGE (Tris/Tricine system: 16% polyacrylamide, 6 M urea).
For detection and quantification of the 129I-TID-PC/16 radioactivity, the gel was subjected to autoradiography using a PhosphorImager (Image-
Quant software, version 3.3) from Applied Biosystems.

RESULTS

Labeling of HA after Incubation of Virus with Liposomes Bearing 129I-TID-PC/16—Consistent with previous studies (7, 8), photolabeling of virus incubated at pH 5 with liposomes containing the nonexchangeable photoactivatable lipid 129I-TID-
PC/16 resulted in selective labeling of the HA2 subunit of HA. Under preincubation conditions (pH 5, 0 °C, 20 s), labeling is ap-
proximately 25 times weaker than that measured upon fusion (pH 5, 37 °C, 10 min). To determine the region(s) labeled within
the HA2 polypeptide chain, the poly peptide was cleaved at tryptophan residues (Fig. 1A) with BNPS-skatole. Thus generated
fragments were separated by SDS-PAGE and, after blotting onto polyvinylidene difluoride membranes, identified by N
terminal Edman degradation. Fig. 1B summarizes the results obtained from cleavage and analysis of both intact HA2
and BHA2. Of particular relevance is the presence in both cleavage products of an (identical) triplet of fragments corre-
mapping to amino acid residues 1–92 (Hsk-5, Bsk-3), 14–92
(Hsk-6, Bsk-4), and 21–92 (Hsk-7, Bsk-5). While Hsk-5(Bsk-3)
contains both the fusion peptide and part of the coiled coil
region, Hsk-6(Bsk-4) lacks most of the fusion peptide.

Under preincubation condition labeling (Fig. 2A and B, lanes 1),
most of the radioactivity was originally associated with HA2 was recovered within Hsk-5. The slight shift of the radioactivity band,
toward higher molecular weight was expected and is due to the
covariantly bound lipid residue. While Hsk-6 may also contain
labeled fragment, Hsk-3, as well as the appearance of low
molecular weight bands, most likely derived from the COOH-
terminal anchor segment. The latter is evident from the heavily
labeled fragment, Hsk-3, as well as the appearance of low
molecular weight bands, most likely derived from the COOH-
terminal part of HA2. Despite the generally higher background
radioactivity, we again note that only the larger band of the
characteristic triplet is labeled.

Labeling of BHA—Next we investigated the interaction at pH
5 of BHA with liposomes containing 129I-TID-PC/16. This
soluble form of HA would be expected to undergo membrane
insertion with particular ease. Following labeling of BHA
and BNPS-skatole fragmentation of the BHA2 poly peptide,
the cleavage products were analyzed by SDS-PAGE and autora-

13418
diography (Fig. 3). Again, we find radioactivity mainly associated with Bsk-3. Neither Bsk-5 nor Bsk-2 are detectably labeled. The second radioactive band corresponds to uncleaved BHA2.

BHA labeled at pH 5 was also subjected to thermolysin cleavage. A single major fragment of molecular mass 19 kDa was generated (Fig. 4). As confirmed by its NH2-terminal amino acid sequence (LKSTQA), this fragment was produced by removal from BHA2 of the NH2-terminal 37 residues and, therefore, corresponds to TBHA2, but lacking the disulfide-bonded, short HA1 segment (23). As evident from Fig. 4, removal of the NH2-terminal region from BHA2 resulted in a complete loss of radioactivity, a result fully consistent with the data from chemical cleavage.

pH-dependent Labeling of Loop-40 Peptide—The absence of label within the coiled-coil region of HA or BHA prompted us to investigate the membrane-binding behavior of a synthetic peptide (loop-40) that corresponds to a segment of HA2 from residue 54 to 93. This segment comprises the loop region and the first part of the long α-helical stem. It is thus identical to those peptides investigated by Yu et al. (10), except that it lacks the amino acid substitutions that had been necessary in the former study to introduce the nitroxide spin label.

For these experiments 125I-TID-BE was used as the labeling reagent. Owing to its smaller size as compared to 125I-TID-PC/16, labeled peptide and lipid could be more easily separated by Sephadex LH-60 gel filtration. Fractions containing loop-40 were analyzed by SDS-PAGE and autoradiography (Fig. 5). As shown in panels A and C, loop-40 was eluted between fractions 22 and 31. As assessed by Coomassie Blue staining, more than 70% of the peptide applied to the column was recovered. The corresponding autoradiographs revealed clear differences in the extents of labeling of loop-40 at pH 7.4 (panel B) and 4.6 (panel D), respectively. Whereas at pH 7.4 loop-40 was labeled only very weakly (less than 0.005% of the original radioactivity), labeling at acidic pH led to clear incorporation of radioactivity into loop-40 (0.025% of the original radioactivity). The heavy radioactivity seen near the bottom at the right-hand sides of the gels originates from 125I-TID-BE-labeled phospholipid which was eluted after loop-40 from the column. Experiments were also performed to compare the extent of labeling of loop-40 with that of BHA. The large excess of lipid (5 mM) over loop-40 or BHA (each 10 mM) provided conditions that allow for (nearly) complete binding of the peptide/protein to the liposomes (10). After labeling at pH 4.6 the specific labeling of loop-40 and that of the BHA2 subunit of BHA were compared. BHA2 was labeled somewhat stronger (0.039% of the original radioactivity) than loop-40 (0.025%). We also confirmed that labeling of BHA by 125I-TID-BE was confined to the fusion peptide (data not shown).

DISCUSSION

In a first, kinetically distinct step in the fusion process, influenza virions adhere to the target membranes. This study now establishes that the sole part of HA2 that inserts into the target membrane is the NH2-terminal fusion peptide. How this hydrophobic peptide can move outwards to the distal tip of the HA2 molecule and become accessible to the target bilayer is implicated by the x-ray structure of TBHA2, an aspect proposed earlier by Carr and Kim (25).

While insertion of the fusion peptide into the target bilayer is a key step in fusion, it does not explain how the two membranes come close together. A possible mechanism has been proposed by Yu et al. (10) on the basis of studies with synthetic peptides.
comprising part of the HA2 polypeptide chain. Following initial attachment of the fusion peptide, some or all of the long coiled coil trimer of HA may insert into the target bilayer, dragging the two fusing membranes into proximity. The results reported here do not support this model. Both during initial attachment and later in fusion, the sole region of the HA2 ectodomain that inserts into the membrane bilayer is the fusion peptide. This
In conclusion, these data suggest that the observed interaction of loop-40 peptide with membranes is unlikely to be relevant to what happens with intact HA during fusion. Membrane insertion of loop-40 as demonstrated by spin labeling experiments (10) and photolabeling may thus be a result of the marginal stability of this relatively short peptide (26).

How then can the two membranes be brought into close proximity? A possible clue to an answer may have come from previous studies suggesting that the HA2 fusion peptide can also insert into the viral membrane (14, 23). Such topological configuration of the HA2 polypeptide chain is not only attained during acid inactivation of virus, but is also a consequence of membrane fusion following initial insertion of the fusion peptide into the target bilayer (27). That HA2 can adopt two distinct topological configurations is not inconsistent with the x-ray structure of TBHA2. In addition to the rearrangement resulting in NH2-terminal extension of the coiled coil, other changes occur in the COOH-terminal part of the molecule which becomes disordered possibly reflecting increased flexibility. This could allow the HA2 coiled coil to adopt two reversed orientations, one in which the fusion peptide is inserted into the target membrane, the other with the fusion peptide in the viral/fused membrane. As discussed previously (14, 27) and depicted schematically in Fig. 6, the reversal of the coiled coil may be directly coupled to membrane merging and fusion. In the absence of a target membrane bilayer, the fusion peptide, after its transient exposure, may insert directly into the viral membrane, representing a plausible mechanism for the inactivation of the virus’ fusion capacity.

Acknowledgment—We thank Dr. John Anagli for the synthesis of loop-40.

REFERENCES

1. Wiley, D. C., and Skehel, J. J. (1987) Annu. Rev. Biochem. 56, 365–394
2. Ohnishi, S.-i. (1988) Curr. Top. Membr. Transp. 32, 257–296
3. Stegmann, T., Doms, R. W., and Helenius, A. (1989) Annu. Rev. Biophys. Chem. 18, 187–211
4. White, J. M. (1992) Science 258, 917–924
5. Zimmerberg, J., Vogel, S. S., and Chernomordik, L. V. (1993) Annu. Rev. Biophys. Biophys. Struct. 22, 433–466
6. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) Nature 289, 366–373
7. Stegmann, T., Delfino, J. M., Richards, F. M., and Helenius, A. (1991) J. Biol. Chem. 266, 18404–18410
8. Tsurudome, M., Gluck, R., Graf, R., Falchetto, R., Schaller, U., and Brunner, J. (1992) J. Biol. Chem. 267, 20225–20232
9. Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Nature 371, 37–43
10. Yu, Y. G., King, D. S., and Shin, Y.-K. (1994) Science 265, 274–276
11. Harter, C., Päch, T., Semenza, G., and Brunner, J. (1989) Biochemistry 28, 1856–1864
12. Harter, C., James, P., Päch, T., Semenza, G., and Brunner, J. (1989) J. Biol. Chem. 264, 6459–6464
13. Brunner, J., Zugliani, C., and Mischer, R. (1991) Biochemistry 30, 2432–2438
14. Weber, T., Paeadoll, G., Galli, C., Mischer, R., Semenza, G., and Brunner, J. (1994) J. Biol. Chem. 269, 18353–18358
15. Weber, T., and Brunner, J. (1995) J. Am. Chem. Soc. 117, 3084–3095
16. Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R., and Brunner, J. (1995) J. Biol. Chem. 271, 17575–17581
17. Gerhard, W. (1976) J. Exp. Med. 144, 985–995
18. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) Biochem. Biophys. Acta 812, 55–60
19. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
20. Ball, E. H. (1986) Anal. Biochem. 155, 23–27
21. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356
22. Konigsberg, W. H., and Henderson, L. (1983) Methods Enzymol. 91, 254–259
23. Wharton, S. A., Calder, L. J., Ruigrok, R. W. H., Skehel, J. J., Steinhauser, D. A., and Wiley, D. C. (1995) EMBO J. 14, 240–246
24. Laurière, M. (1993) Anal. Biochem. 212, 206–211
25. Carr, C. M., and Kim, P. S. (1993) Cell 73, 823–832
26. Hughson, F. M. (1995) Curr. Biol. 5, 265–274
27. Gaudin, Y., Ruigrok, R. W. H., and Brunner, J. (1993) J. Gen. Virol. 74, 1541–1556
28. Tatulian, S., Hinterdorfer, P., Baber, G., and Tam, L. K. (1995) EMBO J. 14, 5514–5523