Hairpin Configuration of H-2K^k in Liposomes Formed by Detergent Dialysis†

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ABSTRACT: H-2K^k is a transmembrane glycoprotein having the N-terminal region of the heavy chain exposed at the cell surface and the C-terminal region exposed at the cytoplasmic face in its native configuration in the plasma membrane. The configuration of H-2K^k in liposomes formed by detergent dialysis was investigated by using fluorescently labeled H-2 and Co^{2+} ions to quench fluorescence. H-2K^k was incorporated into sealed lipid vesicles when deoxycholate was removed by dialysis from a mixture of protein and lipid. Including 20 mM carboxyfluorescein (CF) in the mixture prior to dialysis resulted in CF trapped inside the vesicles at concentrations where self-quenching occurred. Vesicles with CF trapped inside were shown to be osmotically active and impermeable to Na^+ and Co^{2+} ions. In order to examine the configuration of H-2K^k in these liposomes, the heavy chain was covalently labeled by using the sulfhydryl reactive fluorescent reagents fluorescein-5-ylmaleimide (NFM) or 5-[[2-[(iodoacetyl)amino]-ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS). In both cases, approximately equal amounts of fluorescent label were incorporated into the N- and C-terminal regions of the protein. Incorporation of the labeled H-2 into liposomes and examination of the effect of Co^{2+} on the fluorescence showed that all of the label was accessible to quenching by Co^{2+} and thus exposed on the outside of the liposome. The results demonstrate that the H-2K^k is incorporated into these liposomes in a hairpin configuration, not in the transmembrane configuration found in native membranes. Implications of these results for the mode of incorporation of anchored membrane proteins into liposomes by detergent dialysis and for biological studies of H-2 antigen recognition by lymphocytes are discussed.

The class I molecules of the major histocompatibility complex (MHC),[1] HLA-A, -B, and -C in humans and H-2K, D, and L in mice, are glycoproteins present on the surface of most somatic cells. Lymphocyte recognition of these molecules plays a central role in generation of many immune system responses. These proteins are highly polymorphic, and detailed studies of amino acid and nucleotide sequences are revealing the structural basis and extent of this polymorphism [for reviews see Nathenson et al. (1981) and Orr (1982)]. At the same time, the general structural features of these proteins are highly conserved within and between species. They consist of a glycosylated heavy chain of M_r 40 000–50 000 and a noncovalently bound light chain of M_r 12 500 which has been identified as β_2-microglobulin (β_2-M) (Figure 1). The bulk of the protein, including the N-terminus of the heavy chain and all of the β_2-M, is exposed at the cell surface. The exposed region of the heavy chain carries the polymorphic determinants and is accessible for recognition by lymphocytes. The protein is anchored in the membrane by a segment of the heavy chain consisting of about 24 hydrophobic amino acid residues which spans the bilayer. A further segment of about 39 residues, largely hydrophilic and including the C-terminus, is exposed at the cytoplasmic face of the membrane.

One approach to study of the lymphocyte recognition of these antigens has been the use of the purified proteins incorporated into liposomes. Such studies require that the proteins retain their native conformations, and reconstitutions have therefore been done by using detergent dialysis, thus avoiding exposure to organic solvents or extensive sonication. H-2 (Herrmann & Mescher, 1981; Stallcup et al., 1981) and HLA (Engelhard et al., 1978b) antigens incorporated into liposomes in this way retain activity for stimulating generation of cytolytic T lymphocyte responses via specific receptor-mediated recognition of the antigen. Use of these antigen-bearing liposomes has provided an effective means of defining the cellular and soluble factor requirements for generation of the in vitro response [reviewed in Mescher et al. (1982)]. Having defined these requirements, it is now feasible to investigate the molecular requirements for effective recognition by cells. Clearly, such investigation will require a detailed knowledge of the configuration of the H-2 antigen in the liposomes.

Dialysis to remove deoxycholate (DOC) from a mixture of lipid and class I antigen, the procedure used to prepare liposomes for most of the biological studies that have been done, results in formation of small unilamellar vesicles (Engelhard et al., 1978a; Herrmann & Mescher, 1981). Association of the antigen with the liposomes requires that the hydrophobic, transmembrane region be present. The trypsin cleavage product of H-2, lacking the C-terminal hydrophilic region (Figure 1), incorporates into liposomes, but the papain cleavage product, lacking the hydrophobic region (Figure 1), does not (Herrmann et al., 1982). Most (>90%) of the liposome-associated antigen is asymmetrically arranged and exposed at the surface, as demonstrated by antibody and protease accessibility. What has remained unclear is whether the incorporated antigen is in a transmembrane configuration in the liposome bilayer as it is in the native membrane. Alternatively, it might be associated with the bilayer via the hydrophobic region but have both the N-terminal and C-terminal regions exposed at the surface in a hairpin configuration. Trypsin treatment of HLA-containing liposomes did not cleave the

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[1] Abbreviations: MHC, major histocompatibility complex; AEDANS–H-2, the fluorescent product of reaction of IAEDANS with H-2K^k; β_2-M, β_2-microglobulin; CF, carboxyfluorescein; DOC, deoxycholate; IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; NFM, fluorescein-5-ylmaleimide; NFM–H-2, the fluorescent product of reaction of NFM with H-2K^k; PBS, phosphate-buffered saline; PBES, phosphate-buffered Earle's salts; PC, phosphatidylcholine; TBS, Tris-buffered saline; TX-100, Triton X-100; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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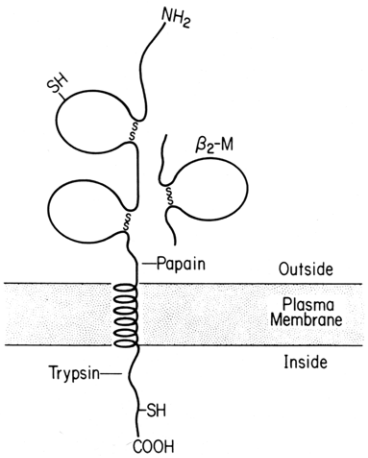

FIGURE 1: Schematic diagram of a class I (H-2K or D) murine MHC antigen. Arrows indicate proteolytic cleavage sites of H-2K^k (Herrmann et al., 1982). Approximate locations of free sulfhydryl groups are those known for H-2K^b (Coligan et al., 1981; Martinko et al., 1981). See text for details.

C-terminus of the heavy chain (Engelhard et al., 1978a), suggesting that it might be transmembrane. As the authors pointed out, however, it was possible that the C-terminus was present at the surface but inaccessible to the enzyme for steric reasons.

The presence of free sulfhydryl groups in both the N-terminal and C-terminal regions of some H-2K and D heavy chains (Coligan et al., 1981; Martinko et al., 1981) suggested an alternative approach to investigation of the configuration of the proteins in liposomes. Attachment of fluorescent probes to each end of the molecule via these sulfhydryl groups and use of a membrane impermeant fluorescence quencher would make it possible to determine if one or both ends are exposed at the liposome surface. A variety of quenching agents is available, but water solubility and charge do not serve as predictors of membrane impermeability (Chalpin & Kleinfeld, 1983). Co^{2+}, however, is an effective quenching agent and is impermeable (Oku et al., 1982; A. M. Kleinfeld, unpublished results). In this report we demonstrate that H-2-containing liposomes formed by DOC dialysis are sealed, osmotically active, and impermeable to Co^{2+}. Furthermore, H-2K^k could be labeled in both the N- and C-terminal regions by using fluorescent sulfhydryl reactive reagents. Addition of Co^{2+} to suspensions of liposomes containing the labeled H-2K^k quenched all of the fluorescence, demonstrating that the heavy chain is incorporated in a hairpin configuration with both the N-terminal and C-terminal regions exposed at the surface. Implications of these findings for the mechanism of protein incorporation by detergent dialysis and for use of these preparations in biological studies are discussed.

Materials and Methods

Purification of H-2K^k. The RDM-4 (H-2^k) murine lymphoma cell line was maintained by weekly passage in AKR or (AKR × DBA2)F_1 mice (Jackson Laboratory, Bar Harbor, ME).[2] Large-scale antigen preparations were done with about 10^{10} RDM-4 cells obtained from 20 mice. H-2K^k was purified by affinity chromatography on a column consisting of the 11-4.1 monoclonal antibody (Oi et al., 1978) coupled to Sepharose 4B as previously described (Herrmann & Mescher, 1979). Protein was assayed by the method of Lowry et al. (1981) in the presence of 1% sodium dodecyl sulfate (SDS) using bovine serum albumin as the standard. Purity of the preparations was assessed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) followed by staining with Coomassie blue to visualize the protein.

^{125}I-H-2K^k was prepared with Iodobeads (New England Nuclear, Boston, MA). Twenty micrograms of H-2K^k was mixed with 0.2 mCi of carrier-free Na^{125}I in 0.2 mL of 0.5% DOC–TBS (Tris-buffered saline; 10 mM Tris, pH 8, and 0.14 M NaCl). One Iodobead was then added and the mixture allowed to stand for 30 min on ice. The reaction mixture was then passed over a Sephadex G-25 column (0.5 × 7 cm) and the excluded peak collected, made 0.5% in TX-100, and dialyzed overnight vs. 2 L of 0.5% TX-100–10 mM Tris, pH 8, followed by 6-h dialysis vs. 0.5% TX-100–10 mM Tris, pH 7.2. The dialyzed sample was then passed over an 11-4.1 monoclonal antibody affinity column and the ^{125}I-H-2K^k eluted (Herrmann & Mescher, 1979). This procedure typically yielded ^{125}I-H-2K^k having 50 000–75 000 cpm/μg of protein. Labeled antigen was stored at −30 °C and used within 3 weeks.

Fluorescent Labeling of H-2K^k. AEDANS–H-2K^k was prepared by labeling affinity-purified H-2K^k. H-2K^k at 0.35 mg/mL in 0.5% TX-100–0.05 M NaCl–10 mM Tris, pH 8, was made 2 mM in DTT and incubated 45 min at room temperature. Solid IAEDANS (Molecular Probes, Junction City, OR) was added and dissolved in the dark to give a final concentration of 7.4 mM. The solution was incubated for 45 min at room temperature and β-mercaptoethanol then added to a final concentration of 13 mM to quench the reaction. The mixture was dialyzed overnight vs. 0.5% TX-100–10 mM NaCl–10 mM Tris, pH 7.2, and a trace of ^{125}I-H-2K^k was then added and the AEDANS–H-2K^k purified by affinity chromatography.

Attempts to label purified H-2K^k in detergent solution using fluorescein-5-ylmaleimide (NFM) (Molecular, Probes, Junction City, OR) were unsuccessful (see Results). NFM–H-2K^k was therefore prepared by reacting whole cells or membranes with the reagent and then solubilizing and purifying the labeled H-2K^k. Whole cells were labeled by suspending freshly harvested and washed RDM-4 cells in PBS (phosphate-buffered saline; 0.14 M NaCl and 10 mM sodium phosphate, pH 7.2) at 1.5 × 10^8 cells/mL with 1 mM NFM. The reaction was allowed to proceed for 1.5 h at 4 °C in the dark with constant stirring. The cells were then pelleted by centrifugation at 1000g for 5 min and washed twice with PBS. The washed cells were then solubilized in 0.5% TX-100–20 mM sodium phosphate, pH 7.2, at 5 × 10^8 cells/mL, and a trace of ^{125}I-H-2K^k was then added and the H-2K^k purified by affinity chromatography.

NFM–H-2K^k was also prepared by labeling membranes from RDM-4 cells, prepared as previously described (Lemonnier et al., 1978). Membranes from 3.5 × 10^8 cells were suspended in 1 mL of PBES (phosphate-buffered Earle's salts) with 10 mM NFM. The reaction was allowed to proceed for 1 h at room temperature in the dark with constant stirring. The suspension was then cooled to 4 °C, and the membranes were pelleted by centrifugation for 30 min at 22000g. The membrane pellet was solubilized by resuspending in 0.5% TX-100–20 mM sodium phosphate, pH 7.2 at 4 °C for 20 min. The mixture was then centrifuged 45 min at 100000g to remove insoluble material, a trace of ^{125}I-H-2K^k added to the supernatant, and the labeled H-2K^k purified by affinity chromatography.

[2] "Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, revised 1978)".

In each case, the stoichiometry of labeling was estimated by comparing the fluorescence intensity of the labeled H-2K^k to that of known concentrations of the cysteine adduct of NFM or IAEDANS in the same buffer. Protein concentration was determined by the method of Lowry et al. (1951) in the presence of 1% SDS using bovine serum albumin as the standard.

Location of Fluorescent Label in H-2K^k. The relative amounts of fluorescence in the N- and C-terminal regions of labeled H-2K^k were determined by comparing the fluorescence intensity of heavy chain before and after removal of the C-terminus by papain cleavage (Herrmann et al., 1982). Some degradation of heavy chain occurs during papain cleavage. ^{125}I-H-2K^k isolated from cells surface labeled by lactoperoxidase (LPO) catalyzed iodination (Herrmann & Mescher, 1979) was therefore included to allow determination of recovery following cleavage. LPO-catalyzed iodination of cells labels H-2K^k heavy chain only in the N-terminal region, and any decrease in ^{125}I radioactivity of the heavy chain thus represents loss due to degradation to lower molecular weight material.

Five micrograms of fluorescently labeled H-2K^k and 10000 cpm of ^{125}I-H-2K^k were mixed in 0.25 mL of 0.5% DOC–TBS, pH 8, and 0.25 mL of 28 μg/mL papain (Sigma, St. Louis, MO) in 0.5% TX-100–TBS with 5 mM cysteine was added. The papain was activated immediately before use by incubation at 2.8 mg/mL with 20 mM cysteine for 30 min at 37 °C. A control sample of labeled H-2K^k was prepared identically but without papain present. The samples were incubated at room temperature for 30 min then made 10 mM in iodoacetamide and incubated for 5 min at 100 °C to stop the reaction. Protein was precipitated by addition of 1 volume of ethanol and 5 volumes of acetone and incubated overnight at −20 °C.

Precipitates were dissolved in sample application buffer and electrophoresed on SDS–polyacrylamide slab gels by using the buffer system described by Laemmli (1970) with a 3% polyacrylamide stacking gel and a running gel consisting of a 5–15% gradient of polyacrylamide. This gel system gives good resolution of the native and papain-cleaved heavy chains (Herrmann et al., 1982). Following electrophoresis, lanes containing the samples were scanned for fluorescence on a Schoeffel SD 3000 microspectra densitometer. Excitation wavelengths of 430 and 350 nm were used for NFM– and AEDANS–H-2K^k, respectively. Lanes containing nonfluorescent standard proteins were also scanned to ensure that light scattering was not contributing to the measured fluorescence intensity. Fluorescence was measured as total peak area for the heavy chain.

After fluorescence was determined, the gels were stained with Coomassie blue and examined to confirm that conversion from native heavy chain to the cleavage product was complete for the papain-treated samples. The heavy chain bands were then cut from the gel, and ^{125}I radioactivity was determined. Recovery of the papain cleavage product ranged from 60 to 80% of the untreated sample. The measured recovery was used to correct the fluorescence value of the cleavage product.

Formation of Liposomes. Lipids used for liposome formation were obtained by chloroform–methanol extraction of tumor cells as previously described (Herrmann & Mescher, 1981), the same lipid preparation used in biological studies of H-2 recognition on liposomes. The washed lipid extract was dried under an N_2 stream and dissolved in 0.5% DOC–TBS, pH 8, to give a final concentration of about 3 μmol of phosphate/mL and stored at −20 °C. Organic phosphate was determined by the method of Ames (1966). Amounts of lipid used for preparations are expressed in moles of phosphate.

Liposomes were prepared by mixing the protein and lipid components in 0.5% DOC–TBS, pH 8, with H-2K^k at a final concentration of 20 μg/mL. The mixtures were dialyzed at 4 °C for 24 h vs. 4 L of TBS, pH 8, followed by an additional 4 L of TBS, pH 8, for 24 h. Trace amounts of ^{125}I-H-2K^k or [^{3}H]phosphatidylcholine (PC) (27 Ci/mmol; New England Nuclear, Boston, MA) were added in some experiments to monitor protein and lipid. In cases where both labels were used, at least a 10-fold excess of [^{3}H]PC radioactivity over ^{125}I-H-2 radioactivity was added and the ^{3}H cpm determined by scintillation counting was corrected for the small (<15%) contribution due to ^{125}I. Liposomes containing trapped carboxyfluorescein (CF) were prepared as described above but with 20 mM CF added to the starting mixture.

In some experiments, liposomes were examined on dextran gradients (Steck, 1974). Dextran (Sigma Chemical Co., St. Louis, MO) with an average molecular weight of 70300 was used, and solutions were prepared in TBS. Centrifugation was done in 18-mL tubes in a Beckman SW27 swinging bucket rotor for 16 h at 25000 rpm. Following centrifugation, 0.5-mL aliquots were collected from the bottom of the tube.

Fluorescence Measurements. Fluorescence measurements were made with a modified Perkin–Elmer MPF–2A fluorescence spectrophotometer (Kleinfeld et al., 1979) with excitation and emission slits set at 4 nm. Measurements were carried out in quartz microcuvettes with sample volumes of 0.14–0.4 mL. CF- and NFM-labeled samples were excited at 485 nm and emission spectra scanned from 490 to 600 nm. AEDANS-labeled samples were excited at 350 nm and emission spectra scanned from 420 to 600 nm. Relative intensities were measured at fluorescence peaks, and the data were adjusted for vesicle scattering and dilution due to additions. Changes in emission intensities secondary to gain changes were normalized by comparing the fluorescence intensities of known quinine sulfate concentrations in 0.1 M H_2SO_4 with excitation at 352 nm and emission scanned from 360 to 600 nm. Affinity column elution profiles of labeled H-2 were routinely monitored by using a Farrand Model A fluorometer with a 5840 exciation filter and a 4-64 emission filter.

In experiments to determine the effects of NaCl, $CoCl_2$ or TX-100 addition on liposome-associated fluorescence (trapped CF or fluorescent H-2), the initial fluorescence intensity of the sample was measured. The cuvette was then removed from the fluorometer, the reagent added and mixed, the sample returned to the fluorometer, and the fluorescence again measured. The time from reagent addition to measurement was 1 min or greater. In every case, the change was complete at this time, and subsequent measurements showed no further change.

Results

Permeability Properties of H-2K^k Containing Liposomes. Use of H-2K^k labeled with fluorescent probes to investigate the configuration of the protein in liposomes requires that all of the protein in the preparation be incorporated into sealed liposomes. Dialysis of a mixture of lipid and H-2K^k (Herrmann & Mescher, 1981) or HLA (Engelhard et al., 1978a) in 0.5% DOC results in the formation of small, unilamellar liposomes with associated proteins. Liposomes were prepared in this way by using a mixture of ^{125}I-labeled H-2K^k, cell lipid, and [^{3}H]PC at a lipid to protein ratio of approximately 210:1 (moles of phospholipid:mole of protein). Dialysis was done for 48 h vs. two 4 L volumes of TBS. The dialyzed sample was then applied to the top of a linear dextran gradient (1.00–1.05 g/cm^3) and centrifuged overnight. Examination

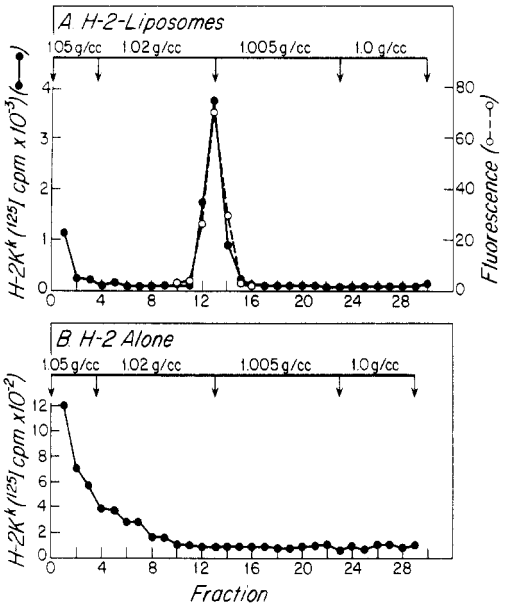

FIGURE 2: Carboxyfluorescein trapping in H-2K^k liposomes. (A) A mixture of 15 μg of H-2K^k, 85 nmol of cell lipid (340:1, mol of phospholipid:mol of protein) and 20 mM carboxyfluorescein in 0.7 mL of 0.5% DOC–TBS was dialyzed as described under Materials and Methods to form liposomes. Dialyzed samples were mixed with an equal volume of 1.1 g/cm^3 dextran and placed in the bottom of tubes, and steps of decreasing density of dextran (as indicated) were layered on top. Samples were centrifuged for 16 h at 25 000 rpm in a Beckman SW27 rotor and 0.5-mL fractions then collected from the bottom of the tubes. (B) H-2K^k dialyzed as in (A) but without added lipid. ^{125}I-H-2K^k; (O) fluorescence units of carboxyfluorescein.

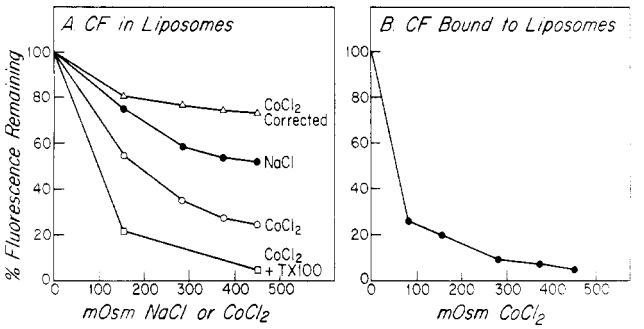

FIGURE 3: Fluorescence quenching of carboxyfluorescein associated with H-2K^k liposomes. (A) Carboxyfluorescein trapped inside liposomes. Liposomes were prepared and isolated on a dextran step gradient as in Figure 2. Fractions containing liposomes were pooled and diluted with TBS to a concentration appropriate for fluorescence measurements. Fluorescence was measured upon addition of increasing concentrations of $CoCl_2$ (O) or NaCl (●). $CoCl_2$ was added as aliquots of 0.4 M $CoCl_2$ in TBS and NaCl as 0.6 M in TBS (i.e., final NaCl at 0.74 M). Fluorescence was corrected for dilution occurring upon salt addition. Triton X-100 was added to a final concentration of 0.5% (w/v) by using a 10% solution in TBS (□). Corrected quenching by $CoCl_2$ (Δ) was calculated by subtracting out the quenching caused by NaCl at the same osmolarity. (B) Cobalt quenching of carboxyfluorescein trapped on the outside of liposomes. Liposomes were prepared as described above with the exception that carboxyfluorescein was not added to the mixture until after 24 h of dialysis.

of fractions from the gradient showed a single coincident peak of protein and lipid (not shown) at a density of 1.006–1.014 g/cm^3. Essentially identical results were obtained with liposomes made by using a 560:1 ratio of lipid to protein. Thus, it appears that all of the H-2K^k is incorporated into lipid vesicles. The position of the liposomes in the dextran gradient suggested that they are sealed to the M_r 70 300 average dextran, since liposomes made at similar lipid to protein ratios have a density of greater than 1.02 g/cm^3 when examined on sucrose gradients (Herrmann & Mescher, 1981; Engelhard et al., 1978a).

Use of Co^{2+} quenching to examine the arrangement of fluorescently labeled H-2K^k in liposomes requires that the vesicles be sealed to ions. Permeability of the vesicles was examined by trapping carboxyfluorescein (CF) in the liposomes during formation. Although CF equilibrates rapidly across a dialysis membrane when dissolved in buffer, we found that the rate of CF removal from samples containing CF in 0.5% DOC was much slower (discussed below in more detail), thereby allowing high concentrations of CF to be trapped within the vesicles. Liposomes were formed by dialysis of a mixture containing 20 mM CF, lipid, and H-2K^k in 0.5% DOC. Flotation of the resulting liposomes on a dextran step gradient (Figure 2A) revealed a single peak of ^{125}I-H-2K^k at the 1.005–1.020 g/cm^3 interface and a coincident peak of CF fluorescence. In separate experiments where [^{3}H]PC was included, all the lipid was found at this same position. When lipid was omitted from the mixture, the H-2 remained at the bottom of the gradient (Figure 2B).

Gradient fractions containing liposomes were pooled and the liposomes examined to determine if CF was trapped within the vesicles. Weinstein et al. (1977) have demonstrated that CF exhibits considerable self-quenching when trapped in vesicles at concentrations greater than 10 mM, and this appears to be the case for the liposomes containing H-2K^k. Addition of TX-100 to free CF in solution caused no change in fluorescence intensity, but addition of TX-100 (final concentration 0.5%) to liposomes containing trapped CF resulted in a 77% increase in fluorescence intensity. This result indicates that CF is trapped inside the liposomes at a self-quenching concentration. Addition of TX-100 results in lysis of the liposomes, thus lowering the effective concentration of CF and eliminating self-quenching. Addition of NaCl to a suspension of the intact liposomes resulted in a concentration-dependent decrease in the CF fluorescence intensity (Figure 3), and subsequent addition of TX-100 to the liposomes in high salt increased the fluorescence to 177% of the initial intensity. These results are consistent with NaCl acting to osmotically shrink the liposomes, resulting in increased self-quenching which is then eliminated by lysis of the vesicles with TX-100. Thus, the H-2K^k-containing liposomes are sealed to both CF and NaCl.

These same vesicles were used to examine permeability to Co^{2+} by determining the amount of CF accessible to Co^{2+}. Co^{2+} is an effective quencher of CF fluorescence in aqueous solutions of CF, and as expected, addition of Co^{2+} to liposomes that had been lysed with TX-100 resulted in almost complete quenching of the CF fluorescence (Figure 3A). If the H-2K^k-containing liposomes are impermeable to Co^{2+}, a significant fraction of the CF fluorescence should remain unquenched in the presence of external Co^{2+}. As shown in Figure 3, addition of Co^{2+} caused a decrease in fluorescence intensity of the intact liposomes in a saturable fashion. At saturation (450 mosm) the fluorescence was about 24% of its initial value. Most of this decrease could be accounted for, however, by $CoCl_2$-induced osmotic shrinkage of the liposomes with a resulting increase in CF self-quenching. NaCl at the same osmolar concentration (450 mosm) caused a 48% decrease in the fluorescence intensity (Figure 3A). By use of the data obtained with sodium chloride, a corrected Co^{2+} quenching curve was obtained (Figure 3A) which showed that 27% of the CF fluorescence could be quenched.

To examine the possibility that the fraction of liposome-associated CF accessible to Co^{2+} was bound to the vesicle surface, H-2K^k-containing liposomes were prepared by dialysis

in the absence of CF for 24 h, followed by addition of 20 mM CF and dialysis for an additional 24 h. The resulting liposomes were then isolated by dextran gradient centrifugation as in Figure 2, and a coincident peak of ^{125}I-H-2K^k radioactivity and CF fluorescence was obtained. Addition of 450 mosm of Co^{2+} quenched 95% of the CF fluorescence (Figure 3B). Neither NaCl nor TX-100 had an effect on the fluorescence (not shown), indicating that the bound CF was not self-quenched. These results indicate that the liposomes are sealed after 24 h of dialysis and that addition of CF at this time results in CF bound to the surface but not trapped inside the vesicles. These results support the conclusion that the liposomes containing trapped CF (Figure 3A) have some CF bound to the surface which is accessible to Co^{2+}. As this surface-bound CF does not appear to be self-quenched, the accessible fraction would be 27 units of total of 177 units or about 15% of the vesicle associated CF.

Preliminary results indicate that H-2K^k-containing liposomes retained CF and remained sealed to Co^{2+} for several days when stored at 4 °C. In all experiments examining the configuration of H-2 in liposomes, however, the vesicles were used immediately after preparation.

Fluorescent Labeling of H-2K^k. As shown below, H-2K^k can be labeled in both the N-terminal and C-terminal regions by using sulfhydryl-reactive fluorescent compounds, indicating that free sulfhydryls are present in each region. We found, however, that attempts to label affinity-purified H-2K^k in DOC-containing buffers were unsuccessful when NFM or (iodoacetamido)fluorescein (IAF) were used. The reagent was mixed with H-2K^k in solution (see Materials and Methods for details), reaction allowed to proceed for 60 min at room temperature, and the sample then dialyzed overnight vs. 0.5% TX-100. Exchange into TX-100 was necessary to allow repurification of the H-2K^k by affinity chromatography on the MAb column. Repurification resulted in elution of coincident peaks of H-2 and fluorescence, but SDS gel electrophoresis of the protein showed that little or none of the fluorescence was covalently bound to the H-2. The dialysis step following the reaction removed the fluorescent label only slowly and incompletely. This observation suggests that the fluorescein derivatives may form mixed micelles with the detergents and limit accessibility of the reagents to the protein sulfhydryl groups. This could also account for the slow rate of removal of CF by dialysis in the presence of DOC (above) which allowed trapping of the CF in liposomes. Reaction of H-2K^k in DOC with IAEDANS under the same conditions (below) resulted in covalent labeling of the protein. While it may be possible to modify conditions or to use a different detergent and obtain labeling of purified H-2 with fluorescein derivatives, we took the alternative approach of labeling cells or membranes in the absence of detergent, followed by detergent solubilization and affinity purification of the labeled H-2K^k. This approach was successful for obtaining fluorescein-labeled H-2K^k.

RDM-4 cells were treated with NFM as described under Materials and Methods and washed in buffered saline to remove unreacted reagent. The cells were then lysed with 0.5% TX-100, the lysate was passed over an affinity column, and the H-2K^k was eluted. Coincident peaks of H-2K^k and fluorescence were found (not shown). Covalent labeling was confirmed by the finding that addition of SDS (to a final concentration of 1%) to an aliquot of the protein, followed by acetone precipitation (6:1 acetone:sample), resulted in precipitation of greater than 95% of the fluorescence.

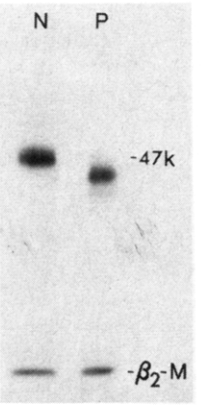

FIGURE 4: Papain cleavage product of NFM–H-2K^k. NFM–H-2K^k containing a trace of ^{125}I-H-2K^k was treated with papain as described under Materials and Methods and electrophoresed on an SDS–polyacrylamide slab gel. After the appropriate lanes were scanned for fluorescence, the gel was stained with Coomassie blue and dried and an autoradiograph prepared. (N) Control, no papain. (P) Papain treated.

Table I: Fluorescent Labeling of H-2K^k: Extent of Modification in N- and C-Terminal Regions[a]

preparation	stoichiometry of labeling (mol of fluor/ mol of H-2)	% of total fluorescence	
		N-terminal	C-terminal
NFM–H-2K^k (whole cell labeling)	0.2	66	34
NFM–H-2K^k (membrane labeling)	0.7	56	44
AEDANS–H-2K^k (protein labeling)	ND	56	44

[a] Proteins were labeled and purified, and the extent of labeling in each end of the heavy chain was determined as described under Materials and Methods.

It was previously shown that treatment of purified H-2K^k with papain under the appropriate conditions removes the hydrophobic membrane spanning region and the hydrophilic C-terminus, leaving an N-terminal M_r 40 600 heavy chain fragment (see Figure 1) that can be recovered with a 70–80% yield (Herrman et al., 1982). NFM–H-2K^k was therefore treated with papain and electrophoresed on an SDS–polyacrylamide slab gel in parallel with an uncleaved sample to determine the percentage of label in the N- and C-terminal regions (Figure 4). Appropriate lanes on the slab gels were then scanned to determine the heavy chain fluorescence before and after papain cleavage. ^{125}I-H-2K^k was included in the initial samples, and the radioactivity that was recovered in the heavy chain following electrophoresis was used to correct for loss due to papain treatment. As shown in Table I, about two-thirds of the fluorescent label was bound to the extracellular, N-terminal region and one-third to the intracellular region.

Fluorescein-labeled H-2K^k was also obtained by treating a crude membrane fraction with NFM, washing to remove the unreacted reagent, solubilizing, and purifying the H-2K^k on the MAb affinity column. The elution profile again showed coincident peaks of ^{125}I-H-2K^k and fluorescence. In this case the extent of labeling was higher, and about half of the label was bound to the C-terminal region (Table I). H-2K^k was also labeled by treating the purified protein in DOC with IAEDANS (see Materials and Methods) and repurifying the labeled protein. Again, the elution profile showed coincident ^{125}I and fluorescence peaks, and in this case also about 50% of the label was in the C-terminus (Table I). These results

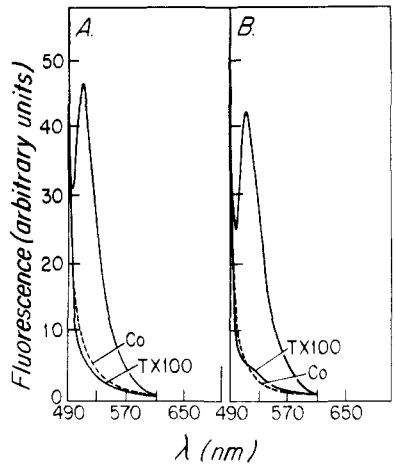

FIGURE 5: Fluorescence intensity quenching of NFM–H-2K^k in liposomes by cobalt. Liposomes were prepared as described under Materials and Methods by using a cell lipid to NFM–H-2K^k ratio of 200:1 (mol:mol). The fluroescence spectra of the liposome suspension were measured without additions (—), with 27 mM $CoCl_2$ (---), and with 27 mM $CoCl_2$–0.5% TX-100 (—). (A) Liposomes containing NFM–H-2K^k obtained by labeling whole cells. (B) Liposomes containing NFM–H-2K^k obtained by labeling membranes.

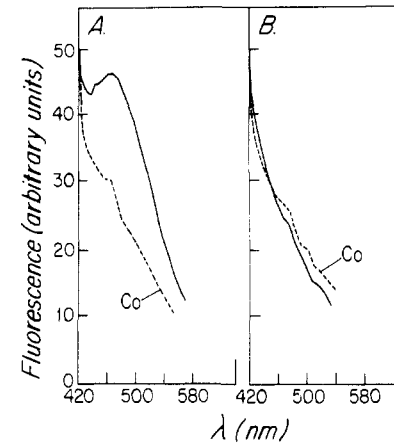

FIGURE 6: Fluorescence intensity quenching of AEDANS–H-2K^k in liposomes by cobalt. Liposomes were prepared by using cell lipid and AEDANS–H-2K^k or unlabeled H-2K^k as described under Materials and Methods. Fluorescent emission spectra were determined before (—), and after (---) addition of 27 mM $CoCl_2$. (A) Liposomes made from lipid and AEDANS–H-2K^k at 200:1 (mol:mol). (B) Liposomes made from lipid and unlabeled H-2K^k at 200:1 (mol:mol).

demonstrate that H-2K^k can be labeled in both the N-terminal and C-terminal regions by sulfhydryl-reactive fluorescent compounds, indicating that H-2K^k, like H-2K^b, has at least one free cysteine in each region.

While both the N-terminal and C-terminal labels contributed about equally to the fluorescence of the heavy chain denatured in SDS (i.e., on SDS–polyacrylamide gels), it was important to rule out the possibility that label in one of the regions might be significantly quenched when the protein is in the native conformation, as it is in liposomes. We therefore compared the fluorescence intensity of the labeled proteins in the absence or presence of 0.5% SDS (heated for 3 min at 100 °C). In the case of AEDANS–H-2, no difference was found for the two samples. In the case of NFM–H-2, fluorescence intensity in SDS was 131% of that in the control. However, the same increase (129%) was seen when the cysteine adduct of NFM was examined in SDS. Thus, the relative amounts of fluorescence in the N- and C-terminal regions of H-2K^k determined on SDS–polyacrylamide gels (Table I) are an accurate measure of the contribution of each label to the fluorescence of the native labeled protein.

Configuration of H-2K^k in Liposomes. Having fluorescently labeled H-2K^k in both ends of the molecule and demonstrated that H-2K^k-containing liposomes are impermeable to Co^{2+}, it was possible to determine, by Co^{2+} quenching, if one or both ends of the H-2K^k heavy chain are exposed at the outside of lipsomes. Examination of Co^{2+} concentration dependence of quenching of the NFM– and AEDANS–H-2 in solution showed that concentrations greater than 20 mM resulted in complete (>95%) quenching (not shown). Experiments with liposomes were therefore done with 27 mM Co^{2+}, a concentration where osmotic effects are minimized.

NFM–H-2K^k was incorporated into liposomes by dialysis at a lipid to protein ratio of 200:1 (mol:mol). Addition of 27 mM Co^{2+} to the liposome suspensions resulted in virtually complete quenching when either whole cell (Figure 5A) or membrane- (Figure 5B) labeled NFM-H-2K^k was used. Subsequent addition of TX-100 to lyse the vesicles resulted in no additional quenching. These results demonstrate that the majority of both the N-terminal and C-terminal regions of H-2K^k are accessible to Co^{2+} and thus present on the liposome surface. If all of the membrane-labeled NFM–H-2K^k were transmembrane, and the C-terminus inside, Co^{2+} would be expected to quench only about 50% of the fluorescence. The observed quenching (>95%) indicates that less than 10% of the H-2K^k could be present in the transmembrane configuration.

It would appear unlikely that the presence of the fluorescein moiety on the relatively large (about 39 amino acid residues) C-terminal hydrophilic region would affect the incorporation or configuration of this region in the liposomes. To further rule out the possibility that NFM affects the configuration of this region, similar experiments were performed with H-2K^k labeled with AEDANS. The lower extinction coefficient and quantum yield of AEDANS in comparison to fluorescein resulted in relatively high backgrounds due to scattering from the liposomes. In order to assess this background, liposomes were prepared in parallel with cell lipid and either AEDANS–H-2K^k or an equivalent amount of unlabeled H-2K^k (mol of lipid:mol of protein = 200:1). Both types of liposomes were diluted identically and examined. Addition of Co^{2+} to the AEDANS–H-2K^k-containing liposomes (Figure 6A) quenched the fluorescence to a level indistinguishable from the background found with unlabeled H-2K^k in liposomes (Figure 6B). Thus, as with NFM–H2K^k, it appears that both the N-terminal and C-terminal regions of AEDANS-labeled proteins are on the outside of the liposomes.

Discussion

H-2K^k was fluorescently labeled in both the N-terminal and C-terminal regions of the glycosylated heavy chain by both NFM and IAEDANS. Incorporation of the H-2K^k into liposomes by dialysis to remove DOC from a mixture of lipid and protein resulted in vesicles impermeable to CF, Na^+, and Co^{2+}. Examination of the effect of Co^{2+} on the fluorescence of labeled H-2K^k in liposomes demonstrated that all of the probe was accessible to quenching by Co^{2+} ions. These results demonstrate that the heavy chain is associated with the bilayer in a hairpin configuration in which the N- and C-terminal regions are exposed at the outer surface (Figure 7b) and not in the transmembrane configuration found in native membranes (Figure 7a).

H-2K^k could be fluorescently labeled to an equal extent in both the N-terminal and C-terminal regions with the fluorescent sulfhydryl reagents NFM or IAEDANS, suggesting that it has at least one free cysteine residue in each region,

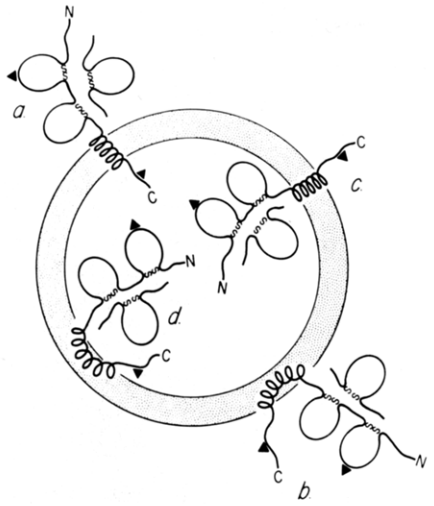

FIGURE 7: Possible arrangements of H-2K^k in liposomes formed by detergent dialysis. (The protein molecules are not drawn to scale with respect to the liposome or bilayer dimensions.)

as does the H-2K^b protein which has been completely sequenced (Coligan et al., 1981; Martinko et al., 1981). The labeled proteins retained serological activity as demonstrated by purification by monoclonal antibody affinity chromatography. Purified H-2K^k in detergent solution could be labeled with IAEDANS but not NFM. Fluorescein labeling of H-2K^k was obtained only when whole cells or membranes were treated with the reagent in the absence of detergents. It appears likely that the fluorescein moiety forms mixed micelles with detergent and is thus not available for reaction with the protein. This might also explain the slow rate of CF dialysis in the presence of DOC, which made it possible to trap CF at high concentration in the liposomes. These observations suggest a significant interaction of fluorescein reagents with detergents, an important consideration in attempts to label purified membrane proteins.

The ability to trap CF in liposomes at high concentrations by the dialysis procedure allowed assessment of vesicle permeability to ions. Changes in fluorescence of liposome-associated CF resulting from increased NaCl concentration or lysis by TX-100 indicated that the majority of the CF was trapped inside the vesicles and that the vesicles were impermeable to Na^+. The liposomes were also impermeable to Co^{2+}, as demonstrated by the inabilitay of Co^{2+} to quench the fluorescence of entrapped CF. Inaccessibility of vesicle-entrapped Calcein to Co^{2+} quenching was previously shown to provide a method of assessing trapped volume (Oku et al., 1982). Since Calcein chelates Co^{2+}, much lower concentrations of Co^{2+} (less than 50 μM) could be used in those experiments. Our results demonstrate that vesicle integrity is maintained at Co^{2+} concentrations 2–3 orders of magnitude greater, thus extending this approach to use of nonchelating fluorescent compounds.

After it was established that H-2K^k-containing liposomes were impermeable and that the protein could be labeled in both the N-terminal and C-terminal regions, it was then possible to examine the configuration of the protein in liposomes. The most likely arrangements for H-2K^k in vesicles are shown in Figure 7. Because removal of the C-terminal hydrophilic region with trypsin does not prevent incorporation, and since the papain cleavage product lacking the hydrophobic region does not incorporate into liposomes (Herrmann et al., 1982), the likely configurations are those having the hydrophobic region inserted into the liposome bilayer. Previous work had demonstrated that H-2K^k (Herrmann & Mescher, 1981) or HLA (Engelhard et al., 1978a; Curman et al., 1980) in liposomes is asymmetrically arranged, with the N-terminal region exposed and accessible to antibody or proteases. Thus, configurations shown in parts c and d of Figure 7 cannot be correct for the majority of the incorporated protein. The results reported here which demonstrate that fluorescent labels in both the N-terminal and C-terminal regions are accessible to quenching by Co^{2+} show that the majority of the protein (>90%) is arranged in the liposomes as shown in part b (Figure 7). A transmembrane configuration, as shown in Figure 7a, would have allowed only 50% of the fluorescence to be quenched by Co^{2+}. Thus, it appears that H-2K^k incorporation into liposomes by detergent dialysis results in a hairpin configuration with both the N-terminus and C-terminus exposed at the vesicle surface. The identical results obtained with very different fluorescent labels, AEDANS and fluorescein, make it unlikely that the hairpin configuration results from an effect of the label on the incorporation. Fluorescent labeling of the protein following reconstitution, and subsequent examination of quenching by Co^{2+}, would more directly address this point. This approach was precluded, however, by the inability to distinguish between fluorescence due to the probe covalently bound to the protein vs. that due to probe nonspecifically bound to the liposomes (as in Figure 3B).

A major impetus for studying the incorporation of MHC antigens into liposomes has been the potential for use of these artificial membranes in investigations of antigen recognition by lymphocytes. Despite the fact that H-2K^k has a hairpin configuration in liposomes, the antigen is effectively recognized in vitro by T lymphocytes (Herrmann & Mescher, 1981; Stallcup et al., 1981; Mescher et al., 1982). It appears likely that the region of the H-2 heavy chain polypeptide immediately above the bilayer has a relatively extended structure, since papain selectively cleaves at this site leaving the rest of the heavy chain and β_2-microglobulin intact (Coligan et al., 1981). Flexibility in this region might allow for association with the bilayer in either a hairpin or transmembrane configuration without having a major effect on orientation of the bulk of the molecule with respect to the plane of the membrane. It is possible, however, that the hairpin configuration results in quantitatively less efficient recognition.

A hairpin configuration for H-2K^k in liposomes appears consistent with the proposal that lipid vesicles form during dialysis at an intermediate detergent concentration when the proteins are still in solution (Curman et al., 1980; Eytan, 1982). The protein would then insert into the already formed bilayer as additional detergent is removed. This mechanism could account for the "right-side-out" orientation of the protein and for the hairpin arrangement, since a transmembrane arrangement would require transfer of the large, predominantly hydrophilic C-terminus across the bilayer. Consistent with this proposed pathway for liposome formation are the observations of Curman et al. (1980) and Cartwright et al. (1982). It was found that addition of HLA or H-2 in detergent to preformed vesicles, under conditions where a large dilution of detergent occurs and the lipid vesicles remain intact, resulted in incorporation of the proteins into the liposomes. It is likely that in this case also the liposome-associated protein has a hairpin configuration.

Trypsin cleavage of HLA antigen in liposomes was examined in an attempt to determine its arrangement with respect to the bilayer (Engelhard et al., 1978a). Cleavage at the trypsin-sensitive site in the C-terminal region was not observed, suggesting a possible transmembrane configuration. As the authors pointed out, however, the possibility could not be

excluded that the C-terminus was at the surface but inaccessible to the enzyme for steric reasons. HLA has been specifically labeled in the C-terminal region with fluorescent probes (Pober et al., 1981), and the method described here could be used to investigate its configuration in liposomes. Viral glycoproteins have also been incorporated into liposomes by detergent dialysis (Helenius et al., 1977; Petri & Wagner, 1979). Again, the proteins were found to be asymmetrically oriented in a right-side-out configuration. Whether or not the proteins spanned the bilayer was not examined.

As discussed above, the hairpin configuration would appear to be the likely result if liposome formation by dialysis involves formation of the lipid vesicles first and then insertion of the protein. Methods of vesicle formation that might be expected to alter the kinetics of these events such as detergent removal from protein and lipid mixtures by gel filtration or rapid dilution might yield different results. If formation of the bilayer and association of the protein with lipid occurred simultaneously, at least some of the protein might have a transmembrane configuration. The kinetics of bilayer formation and protein insertion during dialysis might also be altered to yield some proteins with a transmembrane configuration by varying the detergent used, the lipid composition of the mixture, or the rate of detergent removal. The methodology described in this report will provide a means to explore these possibilities. Another possibility is suggested by studies of the M13 coat protein, a transmembrane protein, by Wickner and colleagues [reviewed in Wickner (1983)]. Their findings have led to the suggestion that an appropriate membrane potential might allow transfer of a hydrophilic polypeptide sequence across the membrane bilayer. Evidence has also been obtained for voltage-dependent translocation of the asialoglycoprotein receptor across a lipid membrane (Blumenthal et al., 1980). Since the H-2K^k-containing liposomes described in this report appear tightly sealed to ions, it should be possible to determine if changes in membrane potential can allow for translocation of the C-terminal hydrophilic region across the bilayer.

Although reconstitution of H-2K^k by detergent dialysis does not result in a transmembrane configuration, similar procedures with other proteins do. This is clearly true for a number of transport proteins [reviewed in Eytan (1982)], one of the best characterized being bacteriorhodopsin (BR). Unlike H-2K^k, BR has multiple hydrophobic regions which span the bilayer. For proteins of this type, association of the protein with lipid may occur simultaneously with formation of the bilayer. Alternatively, the protein may insert into an already formed bilayer, but with the insertion of multiple hydrophobic regions providing the driving force for the movement of hydrophilic regions across the bilayer. This mechanism would be consistent with the observation of Bayley et al. (1982) that BR was asymmetrically oriented in vesicles formed by dialysis from DOC. Engelman & Steitz (1981) have discussed the possibility that insertion of hydrophobic α-helical segments of proteins might provide the driving force for translocation of hydrophilic residues across a bilayer. Thus, the results described here for H-2K^k may be applicable to other anchored membrane proteins having a single membrane spanning region such as HLA, glycophorin, and viral glycoproteins but not to proteins that interact more extensively with the hydrophobic region of the bilayer such as BR and other transport proteins.

Cell surface proteins such as H-2K^k are inserted into the membrane in a transbilayer configuration during synthesis and do not normally exist in a hairpin arrangement. Some proteins, however, may insert into membranes in this configuration. Middle T antigen of polyoma virus is synthesized without a leader sequence (Benjamin, 1982) and is therefore probably translated on free ribosomes. The protein has a hydrophobic stretch of 22 amino acids near the C-terminus, followed by a C-terminal hydrophilic stretch. Once translated, it becomes associated with the inner face of the plasma membrane via this hydrophobic region. Attempts to demonstrate a transmembrane orientation of middle T have been unsuccessful. It would appear likely that its insertion results in a hairpin configuration similar to that found for H-2 in liposomes. A hairpin configuration has also been found for cytochrome b_5 in membranes (Daily & Strittmatter, 1981).

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Polyhistidine Mediates an Acid-Dependent Fusion of Negatively Charged Liposomes†

Chen-Yen Wang and Leaf Huang*

ABSTRACT: Polyhistidine facilitates the fusion of negatively charged liposomes prepared by sonication. Liposome fusion was demonstrated by (a) negative-stain electron microscopy, (b) gel filtration, and (c) resonance energy transfer of the fluorescent phospholipids. Liposome fusion required the presence of polyhistidine, whereas histidine at equivalent concentrations had no effect. Little or no liposome fusion was detectable at pH 7.4, but it was greatly enhanced when the pH of the medium was reduced below 6.5. Although acidic phospholipid is necessary for fusion, liposomes made of acidic lipids alone showed only low levels of fusion activity. Liposomes composed of mixtures (1:1) of a negatively charged phospholipid and a neutral phospholipid such as phosphatidylcholine and phosphatidylethanolamine (PE), but not sphingomyelin, showed high levels (about 80%) of fusion competency. For liposomes made of PE/phosphatidylserine (PS) (1:1), fusion at pH 5.2 and 2.5 μg/mL polyhistidine resulted in an increase in the average liposome diameter from 296 to 2400 Å, indicating multiple rounds of fusion had occurred. Liposome fusion was not very leaky as revealed by the lack of release of encapsulated calcein. For PE/PS (1:1) liposomes, about 10% of dye leakage was observed for up to about 30% liposome fusion and about 45% leakage at 80% liposome fusion. Since polyhistidine becomes a strong polycation at acidic pH, liposome fusion may be a direct result of the bilayer phase separation induced by the binding of polyhistidine with the negatively charged phospholipids. Therefore, this phenomenon is similar to the liposome fusion induced by other polycations such as polylysine at neutral pH. This system may serve as a model for studies on the acid-dependent fusion of viral envelopes with target cell membranes, such as in the case of the influenza, vesicular stomatitis, and Semliki Forest viruses.

Membrane fusion has been extensively studied with liposome model membranes [for reviews, see Papahadjopoulos et al., (1979) and Nir et al. (1983)]. In most of the studies to date, divalent cations such as Ca^{2+} have been used to induce fusion of negatively charged liposomes, e.g., those composed of phosphatidylserine (PS).[1] The evidence points to a mechanism in which Ca^{2+} plays a dual role: it promotes liposome aggregation (Nir et al., 1980) and induces a lipid phase separation which results in fusion (Newton et al., 1978). The concentrations of Ca^{2+} required to induce liposome fusion are generally much higher than the intracellular Ca^{2+} concentrations which trigger the fusion of intracellular membranes, raising some doubt about the physiological relevance of the model studies. Recent studies, however, have shown that in the presence of proteins such as synexin (Hong et al., 1981) and polyamines (Schuber et al., 1983) much lower Ca^{2+} concentrations are necessary to induce fusion.

Effects similar to those observed with divalent cations have been observed with cationic proteins and polypeptides such as polylysine (Hartmann & Galla, 1978). These molecules have the capacity to promote fusion of small unilamellar vesicles containing acidic phospholipids such as PS (Gad, 1983). However, the binding of the polypeptides to the liposomes, in contrast to divalent cations, is practically irreversible (Gad, 1983). Other polypeptides such as polymyxin are also able to induce bilayer membrane fusion. In this case, a lipid–peptide domain is established between the positively charged polypeptide and the negative membrane surface; neutral lipid bilayer membranes are unable to interact with these polypeptides (Sixl & Galla, 1981). Myelin basic protein also has the capacity to induce PG/PC liposome fusion (Lampe et al., 1983). In all of these cases, the involvement of lipid phase separation presumably plays a similarly important role for fusion as in the case of divalent cations.

All except a few (Schenkman et al., 1981; Blumenthal et al., 1983; Connor et al., 1984) of the published model studies have described liposome fusion at neutral pH. Recently a new class of membrane fusion which takes place at a mildly acidic condition has been demonstrated for the fusion of viral envelopes with target membranes (Maeda et al., 1981; White et al., 1981; Marsh et al., 1982). Although the mechanism of membrane fusion is still obscure, it is clear that the viral

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[1] Abbreviations: PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; CL, cardiolipin; Sph, sphingomyelin; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; PBS, phosphate-buffered saline (547 mM NaCl, 11 mM KCl, 6 mM KH_2PO_4, and 4.5 mM Na_2HPO_4).

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