ANOMALOUS SIDE CHAIN AMIDATION IN PLASMA MEMBRANE PROTEINS OF SIMIAN VIRUS 40-TRANSFORMED LYMPHOCYTES INDICATED BY ISOELECTRIC FOCUSSING AND LASER RAMAN SPECTROSCOPY

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SUMMARY

Plasma membranes isolated from normal hamster lymphocytes and lymphoid cells transformed by SV40[1] have been compared. Isoelectric focussing in 1% Triton X-100/8M urea reveals higher isoelectric points than normal for the non-glycosylated proteins in the membranes of transformed cells. This suggests greater amidation of membrane aspartates and/or glutamates. The focussing patterns also reveal a shift to lower pH for the isoelectric points of most glycosylated proteins suggesting increased silalylation. The Amide I - Amide II laser Raman spectra of the two membrane categories are consistent with greater side chain amidation in the membranes of neoplastic lymphocytes.

INTRODUCTION

As recently reviewed (1) extensive evidence suggests that the plasma membranes of neoplastic cells differ biochemically from those of normal cells. Also, many indirect experimental approaches indicate that cultured fibroblasts neoplastically converted by oncogenic DNA or RNA viruses are at least quantitatively deficient in some membrane or membrane-associated proteins (e. g. 2-8), at the same time acquiring some new protein entities identified as transplantation antigens (9).

To characterize possible protein anomalies in the plasma membranes of hamster lymphocytes neoplastically transformed by SV40 (GD 248 cells; 10) we have compared plasma membranes isolated from these cells (11) and from normal lymphocytes by isoelectric focussing and by laser Raman spectroscopy. Both methods suggest that the membrane proteins of the transformed cells are more highly amidated than those of normal lymphocytes.

[1] Abbreviation: SV40 - simian virus 40.

EXPERIMENTAL

Unless stated otherwise all chemicals used were of highest purity grade available. Triton X-100, 4(hydroxymethyl)-1-piperazinylethane-2-sulfate (HEPES)[1], was obtained from Sigma (St. Louis, Mo.) and acrylamide, N, N'-methylene-bis-acrylamide, N, N, N', N'-tetramethylethylenediamine (TEMED)[1], ammonium persulfate and Coomassie brilliant blue from Bio-Rad laboratories (Richmond, Calif.). Ampholytes (Ampholine pH 3.5-10.0) were purchased from LKB (Upsala, Sweden) and periodate Schiff reagent and paraffin oil (Saybolt Viscosity 125/135), from Fisher (Fair Lawn, N. J.). Gangliosides with different numbers of sialic acid residues were kindly provided by Dr. Gerhard Schmidt. Normal and SV40-transformed (GD 248) lymphocytes from outbred golden Syrian hamsters, were isolated as in (11) and their plasma membranes prepared as in (11) using modification of the method described in (12).

For isoelectric focussing membranes were extracted with 1mM HEPES pH 8.5, 1% in Triton X-100. The membrane pellets were suspended in about 0.3ml HEPES-Triton per 10^9 cells and incubated at 37°C for 15 min. Undissolved material was removed by ultracentrifugation at $1.5 \cdot 10^7$ g · min (10°C; 0.6ml polycarbonate tubes, SW56 rotor; Spinco L 2-65B ultracentrifuge). For volumes smaller than 0.5ml, the samples were overlaid with paraffin oil to prevent tube collapse. The pellets were redispersed in the same volume of HEPES-Triton and the suspensions again centrifuged at $1.5 \cdot 10^7$ g · min. The two supernatant fractions were combined to yield a protein concentration between 2 and 4 mg/ml. The extracts were used within one day (without freezing). More than 90% of the protein of both normal and neoplastic lymphocytes was rendered soluble by the extraction procedure.

Isoelectric focussing in polyacrylamide was carried out using an apparatus described in (13), built by Medical Research Apparatus (Boston, Ma.) and a Buchler (Fort Lee, New Jersey) power supply (Model 3-1155), with both current and voltage regulation. We used thin-walled glass tubes (3mm I. D.), containing 5% acrylamide, cross-linked with 2.5% bisacrylamide. The gels contained 2% ampholytes, 8M urea, 1% Triton X-100 and 10% sucrose. For gel casting, the tubes were closed at the bottom with dialysis membranes (kept in place throughout the focussing to prevent gel extrusion). For twelve 10cm-long gels we combined 1.2 ml acrylamide/bisacrylamide (5%/2.5%) with 0.6 ml ampholytes pH 3.5 - pH 10.0 (40%w/v), 9.6 ml 10M urea, 12% in sucrose and 1.2% in Triton X-100, 0.4 ml deionized water, 0.24 ml 2% ammonium persulfate and 0.030 ml 10% TEMED. The mixture was stirred, degassed, immediately cast and the gels used within 3-4 hr. Before protein application, the gels were cooled to 0°C in the focussing apparatus and pre-electrophoresed for 10 min at 0.25 mA/gel. The cathodal and anodal buffers were 0.03 M NaOH, and 0.05 M H_2SO_4, respectively. The proteins were applied to the cathodal end of the gel. Loading was kept below 0.1 mg protein/gel to avoid protein precipitation. The protein was applied in three aliquots at 25 min intervals. At each occasion the material was applied directly on top of the gel and the volume applied previously replaced. Focussing was started at constant-current regulation using 0.25 mA/gel up to 400V. The run was then continued at 400V for 16 hours. The cooling fluid (25% ethylene glycol, 75% water) was kept at 0°C during focussing. Two blank gels were run in each experiment, one to determine the pH gradient and one was stained with Coomassie blue to measure background ampholyte staining. (14).

To determine pH gradients the gels were cut into 0.5 cm slices. These slices were then suspended in 1.0 ml H_2O and the pH measured after 30 min equilibration. The pH gradients were linear and stable between 12 and 24 h. Before staining the gels were fixed in 10% trichloroacetic acid for 6-8 h, then washed with 25% isopropanol, 10% acetic acid, 65% water for 12 h and with water for 2-3 h. They were then stained for 4 h, using 25% ethanol, 10% acetic acid, 65% water containing

[1] Abbreviations: HEPES - 4(hydroxymethyl)-1-piperazinylethane-2-sulfate; TEMED - N, N, N', N'-tetramethylethylenediamine

0.05% Coomassie blue and 0.5% Cu SO_4 (14). Gels were destained with 10% ethanol, 10% acetic acid, 80% water until all background had been eliminated. For periodate-Schiff staining, the gels were fixed with 10% trichloroacetic acid and then stained as in (15). Stained gels were scanned in a Gilford 2400-S spectrophotometer. The focussing patterns illustrated are representative of 3 separate membrane preparations each from normal and transformed lymphocytes.

For Raman spectroscopy, non-solubilized membranes, pelleted at 1.5 x 10^7 g · min were resuspended in 1mM HEPES, pH 7.5 to a concentration of ∿ 10 mg/ml and the suspension transferred to ∿ 1 mm I. D. Kimex capillaries. Raman spectra were obtained as in (16), using a Ramalog 4 Raman spectrometer (Spex Industries, Metuchen, N. J.) interfaced to an Interdata (Model 70) computer. An Ar^+ laser (Spectra Physics model 164), tuned at 488 nm (300 mW power) was used as an excitation source. A cutoff filter was used to eliminate plasma lines appearing above 1200 cm^{-1}. Raman scattering, detected by a thermoelectrically-cooled photomultiplier (RCA 31034), was recorded in terms of photons/s. The "dark" counts of the photodetector were < 100 counts/s. Raman scattering from the samples gave counts in the order of 10^3-10^4/s. Scanning was done through the computer (loaded with the BIE8D Rama comp Computer Program; Spex Industries). The maximum time and minimum time for each data point were 1s and 0.5s respectively. The photon counts were 10^4-10^5 maximum and 100 minimum. Scanning was incremental between data points and no counts were recorded while the spectrometer was moving between data points. Photon counts were stored in the computer memory during scanning (2-4 scans) and the stored spectra, averaged and smoothed by a least-squares procedure (17; included in the computer program), were ultimately plotted on the Ramalog recorder using appropriate background suppressions and scale expansions. The spectra illustrated are representative of three separate membrane preparations each from normal and neoplastic cells.

RESULTS AND DISCUSSION

Table I summarizes important general properties of our plasma membrane preparations.

Representative isoelectric focussing patterns obtained with Triton X-100 extracts of plasma membranes from normal and SV40 transformed lymphocytes are illustrated in Figure 1. Identical patterns were obtained whether the proteins

Table I Yield and purification of plasma membranes from normal and SV40-transformed lymphocytes, using lactoperoxidase-catalyzed ^{125}I-labelling as extrinsic marker and Na^+, K^+-activated ATPase as intrinsic marker[1].

		^{125}I[2]		Na^+, K^+-ATPase[2]	
	Fraction	TA[3] (% recovery)	SA[4] (purification)	TA[3] (% recovery)	SA[4] (purification)
Normal Lymphocytes	Homogenate	$9.5 \cdot 10^5$ (100%)	$4.8 \cdot 10^4$	122.7	2.0
	Plasma Membrane	$4.0 \cdot 10^5$ (42%)	$4.2 \cdot 10^5$ (11.4)	76.1	31.7 (15.8)
SV40-Transformed (GD 248) Lymphocytes	Homogenate	$2.3 \cdot 10^6$ (100%)	$3.9 \cdot 10^4$	107.5	2.59
	Plasma Membrane	$1.1 \cdot 10^6$ (48)	$5.6 \cdot 10^5$ (14.4)	69.9	43.4 (16.7)

[1] Not more than 5% of the NADH-oxidoreductase activity, 0.2% of the β-glucoronidase activity and 0.6% of succinate dehydrogenase activity found in the total cell homogenate was recovered in the plasma membrane fractions of either normal and transformed cells. [2] Results from three independent experiments; variation of specific activities < ± 15%. [3] TA - total activity in cpm and nmoles inorganic phosphorus liberated · min^{-1}, respectively; material from 10^9 cells. [4] SA - specific activity in cpm · mg protein $^{-1}$ and nmoles inorganic phosphorus liberated · mg $protein^{-1}$, min^{-1}.

were polymerized into the gels or applied to the cathodal end and the banding did not change between 12 and 24 h. These observations indicate that focussing equilibrium has been reached at 12h. Since the Triton X-100 extracts contain more than 90% of the membrane proteins, the focussing patterns are largely representative of the membrane proteins.

The proteins in the membranes from normal cells concentrate between pH 7.0 and pH 4.5, forming eight zones (1-8) with about 40 distinct bands. Component 9 (pH ∿ 3.5) staining weakly with Coomassie blue but strongly with the Schiff reagent is probably glycolipid in nature, since purified gangliosides focus in this area. The membrane proteins from neoplastic lymphocyte cells differ from those of normal cells as follows: (1) The distribution of Coomassie-positive bands vs. pH is spread out towards both the acid and alkaline ends of the gradients. (2) A cluster of weak bands, A, appears between pH 7.5 and 7.8 (arrow). (3) A strong band

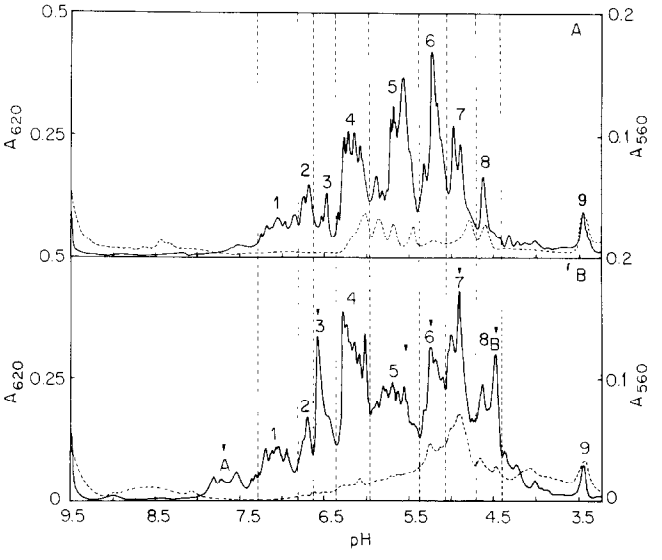

Figure 1. Isoelectric focussing patterns of the plasma membranes of normal lymphocytes, A, and SV40 transformed (GD 248) cells, B, the abscissa gives the experimentally-determined pH gradient. The left ordinates give the absorbance at 620 nm for Coomassie staining (———) and the right ordinates the absorbance at 560 nm for periodate-Schiff staining (------). Vertical dashed lines indicate "focussing zones". Prominent differences in individual bands (zones) are indicated by arrows. A and B refer to components not detected in the membranes of normal cells.

at pH ∿ 6.57 (arrow) with a shoulder at pH 6.5 replaces a weak doublet at pH ∿ 6.5. (4) A set of bands in zone 4 intensifies and a set in zone 5 diminishes. (5) The group of bands in zone 6 diminishes, whereas the set in zone 7 intensifies. (6) A strong band, B, emerges at pH 4.5 (arrow). (7) The distribution of PAS-staining components shifts from the region of pH 6.5-pH 5.4 to the pH range of 5.4 to ∿ 4.0. None of the more than 40 isoelectric components of normal cells are "deleted" in tumor cells and most of the differences between the two membrane types can be ascribed to changes of isoelectric points, with many glycosylated proteins shifting to lower pIs[1] and many non-glycosylated proteins shifting to higher pIs. A possible exception is component B (pI ∿ 4.5) which appears to have no counterpart in the membranes of normal cells.

The simplest explanation for the redistribution of glycosylated components toward acid pH is that in the membranes of tumor cells most or all of these glycoproteins contain more sialic acid than in normal cells, leading to a lower pI. This suggestion fits the observation that neoplastic cells commonly exhibit a greater net negative surface charge than normal cells (18) and that their glycoproteins commonly bear more sialic acid (19).

We can at present suggest only one process to account for the generalized "alkaline shift" of non-glycosylated proteins, namely that a greater proportion of aspartate-glutamate residues is amidated in the membranes of SV40 transformed lymphocytes. Indeed it has already been documented that replacement of one Asn per 22,000 D subunit of muscle aldolase by Asp shifts the pI by 0.9 pH units (20).

Figure 2 depicts the Raman spectra of plasma membranes from normal and SV40 transformed hamster lymphocytes in the Amide I - Amide II region. Table II lists observed band frequencies and tentative assignments. All of the scattering features lie on top of the broad H-O-H' deformation band of H_2O at 1640 cm^{-1} (half-band width = 126 cm^{-1}).

The bands above 1650 cm^{-1} indubitably include contributions from the backbone Amide I vibrations, since plasma membranes are ∿ 70% protein by weight (Table I).

[1] pI - isoelectric point

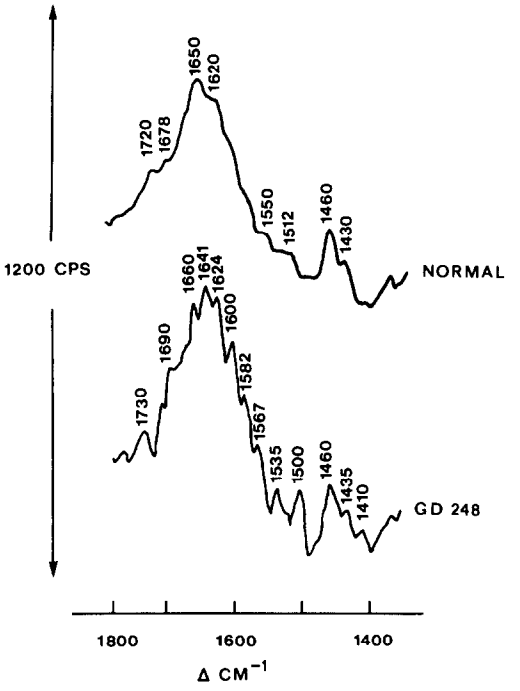

Figure 2. Laser Raman spectra showing the Amide I - Amide II regions of the plasma membranes of normal hamster lymphocytes and GD 248 cells. The ordinate gives scattering intensity in photon counts per second. Excitation at 488 nm, power 300 mW; resolution ∿ 3 cm^{-1}.

Table II Raman frequencies (cm^{-1}) in the Amide I - Amide II region of plasma membranes from normal and SV40 transformed lymphocytes, as well as of several model compounds. [1,2]

Plasma Membranes from Normal Cells	Tumor Cells	Asn	Poly-L-Asn	Gln	Tentative Assignments
1650	1660	1655 sh	1660 s	1662 sh	Amide I (skeletal and residue);
	1641	1640 s	1640 s	1642 s	H_2O; ν C=C (in membranes).
1620 sh	1624	1620 s	1620 s	1615 s	Amide II (residue); dipolar
	1600	1610 sh	1590	1600 sh	antisymmetric CO_2^- stretching in Asn/Gln.
	1582	1580			Trp; Amide II in poly Asn.
	1567	1570	1560		
1550 sh					

[1] Asn, poly-L-Asn and Gln values for aqueous solutions. [2] Bands near 1620 cm^{-1} and the 1600 cm^{-1} band of tumor membranes not detected after deuteration.
ν = stretching; s = strong; sh = shoulder.

However, the features between 1700 cm^{-1} and 1670 cm^{-1}, as well as the band near 1660 cm^{-1}, persist upon deuteration, suggesting that they represent C=C stretching vibrations of *trans*- and *cis* double bonds (21) respectively, in unsaturated phospholipid acyl chains.

The membranes of neoplastic lymphocytes differ consistently from those of normal cells in the following respects: (1) Appearance of defined bands at 1641 cm^{-1}, 1600 cm^{-1}, 1582 cm^{-1}, 1535 cm^{-1} and 1500 cm^{-1}. (2) Replacement of shoulders near 1620 cm^{-1} and 1550 cm^{-1} by defined bands at 1624 cm^{-1} and 1567 cm^{-1}. The differences at $\sim$ 1640 cm^{-1} and 1620 cm^{-1} can be attributed to greater amidation of membrane aspartate/glutamate because (a) these are very prevalent residues and (b) the Amide I and Amide II modes of Asn/Gln as well as poly-L-Asn give strong bands at $\sim$ 1640 cm^{-1} and $\sim$ 1620 cm^{-1} (Table II). Tyr yields a weak feature near 1620 cm^{-1} but the observed difference at this frequency cannot be due to this residue, since the two membranes show no significant difference in the p-hydroxy-phenyl ring band at $\sim$ 836 cm^{-1} (22). Moreover, as expected from an Amide II band, the 1624 cm^{-1} peak of tumor membranes disappears upon deuteration. The band near 1600 cm^{-1} falls into the Amide II frequency range generally of primary alkyl amides (22). That this might be the correct assignment is indicated by the elimination of this peak upon deuteration. It is thus conceivable that the bands at 1624 cm^{-1} and 1600 cm^{-1} represent Asn/Gln residues in different H-bonding environments. The features at 1582 cm^{-1} and 1567 cm^{-1} persist upon deuteration and are most reasonably attributed to the indole ring of Trp (22). The 1500 cm^{-1} band (Fig. 2) is not a consistent feature.

The Raman spectra thus support our interpretation of the "alkaline shift" obtained upon isoelectric focussing as due to greater amidation of Asn/Gln residues in Gd 248 plasma membranes.*

As to possible mechanisms involved, we note that amidases occur in many animal tissues, as well as in the sera of some species (23) and that deamidation of intact proteins can proceed rapidly in vivo (20). Moreover, it is established that the growth of a wide variety of neoplastic lymphocytes can be inhibited by asparaginase (23), that the mitogenic activation of lymphoid cells by diverse lectins can be also inhibited by asparaginase (24). Finally, recent evidence indicates

* Several minor membrane components, e. g. N-acetylated aminosugars, sphingomyelin give Amide bands, but are not present in sufficient amounts to explain the differences between normal and GD 248 cells.

that this inhibition of blastogenesis by asparaginase is due to plasma membrane modifications (25). Our data therefore suggest the possibility that some Asn/Gln residues of lymphocyte membrane proteins are deamidated sometime after biosynthesis and that this process is deficient in the neoplastic cells or in their hosts.

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