Communication

Adenosine Deaminase in Cell Transformation

BIOPHYSICAL MANIFESTATION OF MEMBRANE DYNAMICS*

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Cell transformation is associated with a dramatic collapse of a graphic fingerprint characteristic of normal, as measured by phase fluorimetry. This is demonstrated on adenosine deaminase (ADA, EC 3.5.4.4), an established malignancy marker. ADA activity is known to decrease markedly in chick embryo fibroblasts (CEF) transformed by Rous sarcoma virus. The high affinity between the catalytic small subunit ADA (SS-ADA) and its membranal complexing protein (ADCP) (which abounds on the plasma membrane of CEF) allowed the hybridization of fluorescent labeled SS-ADA with native ADCP on CEF. Multifrequency differential phase fluorimetry responded remarkably to the state of this hybrid membrane protein. The transformation process is shown to have lead to increased membrane fluidity and rotational mobility of ADCP as well as to its reduced availability to SS-ADA binding. The hypothesis of protein vertical sinking into the lipid core of the membrane is now given support by our spectroscopic data. Additional models are considered. A regulatory role is thus suggested for the complexing protein, which may also account for (a) reduced ADA activity in transformed cells and (b) detachment, exclusive to normal cells, upon addition of SS-ADA in excess.

The dynamic nature of biological membranes has been shown to play a major role in a variety of physiological processes (1). Indications of concerted motions of various membranal components mediating a multitude of cell functions have been reported (2). Recently, it was proposed (3) that the activity of membranal enzymes may be controlled by lipid-protein interactions motivating the vertical motion of such enzymes within the lipid core of the plasma membrane. We extended this hypothesis to account for the altered activity of adenosine deaminase after cell transformation (4). The present report provides spectroscopic evidence in line with such a mechanism.

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ADA, an established malignancy marker (5), has a polymorphic tissue-dependent quaternary structure (6-9). It consists of a small 45-kDa catalytic subunit and a large 210 kDa membranal complexing protein, whose physiological function is not yet known. ADCP is thought to anchor SS-ADA to the cell membrane. The high affinity between these subunits prevails even when they originate in different species (6-8). ADCP abounds on the plasma membrane of fibroblasts (9, 10). These properties prompted our choice of ADA for studying the effect of membrane dynamics on enzyme activity.

In order to bridge between the spectroscopic requirements and the inherent complexity of the biological system, several factors had to be combined. 1) To obtain a signal associated specifically with ADCP, we artificially increased the concentration of a large ADA complex by hybridizing the native ADCP on CEF with fluorescent-labeled SS-ADA (from calf intestine). 2) The use of a relatively long lived fluorescent probe, combined with multifrequency phase modulation spectrofluorimetry (13), allowed the opening of a time window from which background "noise" was excluded. 3) The measurement of fluorescence polarization (P) does not depend on signal intensity and is thus unaffected by daily variations, typical to biological systems.

SS-ADA tagged with fluorescein can serve as a histochemical marker for membrane ADCP, while SS-ADA labeled with pyrene would serve for dynamic fluorescence studies.

We report encouraging preliminary results in the study of the hybrid large ADA complex in living cells by means of multifrequency differential phase modulated fluorimetry. Our results indicate increased rotational freedom of hybrid ADA embedded in a fluidized lipid core of the transformed cell membrane. Possibilities of vertical sinking or conformational change of the ADCP in the more fluid membrane are thus implied, with a consequent reduction in ADA activity. In view of the yet unknown physiological role of ADCP, this finding allows us to suggest here a regulatory role for ADCP. The appreciable reduction in ADA activity observed in CEF transformed by RSV (11, 12) may be contributed, at least in part, to this regulatory mechanism. A quantitative reduction in the number of ADCP molecules per transformed cell does not contradict the qualitative change described here.

EXPERIMENTAL PROCEDURES

Materials—All reagents were chemically pure grade or better. ADA of high specific activity (approximately 200 units/mg of protein) from calf intestine was purchased from Sigma as an ammonium sulfate suspension. We estimated it to be at least 90% pure M, 45,000 subunit based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). The ammonium sulfate was removed by dialysis against three changes of 10 volumes of phosphate-buffered saline (154 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4). BSA, purchased from Sigma, was Cohn Fraction V. Sephalex G-25 was purchased from Pharmacia LKB Biotechnology Inc. FITC, pyrene sulfonoyl chloride on celite, DPH, 10-(1-pyrene)-10-ketodecanoic acid, and 12-(9-anthryloxyl) stearic acid were purchased from Molecular Probes Inc. Adenosine was obtained from Sigma. erythro-9-(2-Hydroxy-3-nonyl)adenine was synthesized by us according to an established procedure (15); it had

1 The abbreviations used are: ADA, adenosine deaminase; SS-ADA, small subunit ADA; ADCP, adenosine deaminase complexing protein; BSA, bovine serum albumin; RSV, Rous sarcoma virus; CEF, chick embryo fibroblasts; FITC, fluorescein isothiocyanate; DPH, 1,6-diphenyl 1,3,5-hexatriene.
equivalent inhibition characteristics to that of erythro-9-(2-hydroxy-3-nonyl)adenine received as a gift from Dr. Howard J. Shaeffer, the Wellcome Research Laboratories, Burroughs Wellcome Co.  

Isolation of ADCP—ADCP was isolated from bovine kidney by affinity chromatography, as was already described in detail by us (14).

ADA Assay—ADA activity was followed either by measuring the decrease in absorbance at 265 nm or by the colorimetric method based on phenol/hypochlorite reaction; both techniques were already described by us (4, 14).

Protein Assay—Protein was assayed according to the procedure of Sedmak and Grossberg (16) using BSA as a standard.

Labeling of ADA with Fluorescent Probes—ADA was labeled either with FITC or pyrene sulfonyl chloride. Labeling was carried out by incubating the diazoyl enzyme for 3 h at 4 °C, according to published procedures for FITC (17, 18) and for pyrene sulfonyl chloride (19, 20). The number of fluorescent molecules bound per enzyme was determined by the respective absorbancies and extinction coefficients: for FITC, ε = 78,000 at λmax = 494 nm; for pyrene sulfonyl chloride, ε = 20,000 (21) at λmax = 346 nm. The molar extinction coefficient for pyrene sulfonyl chloride was estimated from its absorbance in water-ethanol mixtures and extrapolated to pure water. The number of enzyme molecules was determined by protein assay, assuming M, = 45,000.

Cells and Viruses—Secondary cultures from 11-day-old chick embryos were prepared and cultivated in M199 medium supplemented by 5% fetal calf serum, as described before (11, 12). RSV and RSV-Ts68 mutant were obtained from Dr. John Bader at the National Cancer Institute. Cells infected with this mutant have transformed morphology at 36 °C and normal morphology at 41 °C. Infection was carried out 1 day after seeding and was completed after two passages.

Fluorescence Measurements—(a) Fluorescence spectra were obtained on the Perkin-Elmer MPF-44 spectrofluorimeter to which an Apple-IIe computer was attached to allow spectral subtraction and multiplications. (b) Steady-state fluorescence polarization measurements were carried out either on the MPF-44 spectrofluorimeter or the SLM 4800, at the respective excitation and emission maxima. (c) Fluorescence lifetime studies were carried out either on the single photon correlation spectrofluorimeter (4) or the SLM 4800 (22). (d) Multiphase modulation spectrofluorimetry was carried out at the Laboratory for Fluorescence Dynamics, University of Illinois at Urbana, according to procedures already described by Gratton et al. (13).

Binding Specificity of FITC-SS-ADA to ADCP on CEF—Secondary CEF at their logarithmic phase of growth were washed and incubated with FITC-SS-ADA (10 units/ml) containing 3 mg/ml BSA for 1 h at 37 °C. Control cells were treated similarly but incubated with 100 units/ml of unlabeled SS-ADA in addition to the FITC-SS-ADA (8). Under these conditions cells remained viable. Following a X4 wash with phosphate-buffered saline containing 3 mg/ml BSA, cells were fixed with 3% formaldehyde. Micrographs were obtained with a Leitz Ortholux 2 fluorescence microscope equipped with an Orthomat camera.

RESULTS AND DISCUSSION

Elution profiles of labeled SS-ADA from a Sephadex G-25 column revealed excellent separation between bound and free excess label. The fluorescence emission of the labeled fractions coincided with their protein content. The label to protein molar ratio was 2 for fluorescein and 1 for pyrene. The labeled enzyme retained both its activity and its affinity to ADCP (isolated from bovine kidney (14)). The affinity of pyrene-labeled SS-ADA to ADCP in solution was deduced from the titration of SS-ADA with an increasing amount of ADCP. Fluorescence polarization increased by a factor of 3 when the ADCP to SS-ADA ratio exceeded the value of 1x4; the corresponding fluorescence lifetime decreased by no more than 20%. The enhanced P values were expected from the formation of a large complex with slower rotational diffusion. Kd = (1.176 ± 0.030) x 10^-5 M was estimated for a 1:1 stoichiometry (M, of ADCP = 200,000).

We found that the fluorescent-labeled SS-ADA specifically binds to ADCP on CEF (Figs. 1 and 2). This was proved by competition with unlabeled SS-ADA in excess (Figs. 1b and 2a) indicating 85% specific binding. The unlabeled enzyme preferentially displaced the labeled enzyme from ADCP binding sites. We have observed patching (Fig. 1a) similar to that found in fluorescent micrographs obtained with specific antibodies against ADCP (8). Binding curves3 flattened at 5 units/ml of FITC-SS-ADA and leveled at 10 units/ml similar to those reported by Andy and Kornfeld (8). We hereby show that CEF do not differ from mammalian fibroblasts in possessing relatively abundant ADCP free to bind externally added SS-ADA.

Unexpected rounding and detachment of normal cells caused by the addition of SS-ADA in large excess (e.g. 100 units/ml) did not occur in transformed cells. Detachment could not be ascribed to a shortage of adenosine, as it took place even in the presence of a high concentration of adenosine or erythro-9-(2-hydroxy-3-nonyl)adenine, a strong ADA inhibitor.

Transformed cells (RSV and RSV-Ts68 (36 °C)) attracted less SS-ADA than normal or "revertant" cells (RSV-Ts68 (41 °C)) (Fig. 2b). Lesser binding was also noted in micrographs of RSV-Ts68 (36 °C) transformed CEF (not shown). Lipid-protein interactions, associated with viral transformation of CEF, were studied independently with pyrene-labeled SS-ADA and with lipophilic probes. Cultured (normal)

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K_d = (1.176 \pm 0.030) \times 10^{-5} \text{M}
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\[
M_r = 200,000
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P = 1x4
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\[M_r = 78,000 \text{ at } \lambda_{max} = 494 \text{ nm for pyrene sulfonyl chloride}
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\[\varepsilon = 20,000 \text{ at } \lambda_{max} = 346 \text{ nm for pyrene sulfonyl chloride}
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FIG. 1. Fluorescence micrographs of (a) FITC-SS-ADA-labeled CEF and (b) their control counterparts. For experimental details see "Experimental Procedures."
Steady state fluorescence polarization with lipophilic probes

Steady state fluorescence polarization measurements with lipophilic probes were carried out on the SLM 4800 spectrophotofluorimeter at 37 °C; \( \lambda_{ex} = 360 \text{ nm} \), \( \lambda_{em} = 430 \text{ nm} \) for DPH, and a 418-nm cut-off filter was used; \( \lambda_{ex} = 347 \text{ nm} \), \( \lambda_{em} = 377 \text{ nm} \) for 10-(1-pyrene)-10-ketodecanoic acid, and a 370-nm cut-off filter was used; and \( \lambda_{ex} = 383 \text{ nm} \), \( \lambda_{em} = 446 \text{ nm} \) for 12-(9-anthroyloxy) stearic acid and a 418-nm cut-off filter was used. Average error is ±5%. Cells were labeled according to published procedures (23, 24). DPH fluorescence lifetime measured by single photon correlation (4) and phase modulation spectrophotofluorimetry (22) was 8.5 ± 0.1 ns for all cells; 10-(1-pyrene)-10-ketodecanoic acid showed a long component lifetime of 10.86 ± 0.14 ns for cells grown either at 36 or 41 °C; a two-lifetime analysis of the SLM 4800 data was employed.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Growth temperature °C</th>
<th>1,6-Diphenyl-1,3,5-hexatriene</th>
<th>10-(1-Pyrene)-10-ketodecanoic acid</th>
<th>12-(9-Anthroyloxy) stearic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>36</td>
<td>0.245</td>
<td>0.164</td>
<td>0.125</td>
</tr>
<tr>
<td>RSV-Ts68</td>
<td>36</td>
<td>0.189</td>
<td>0.100</td>
<td>0.158</td>
</tr>
<tr>
<td>RSV-Ts68</td>
<td>41</td>
<td>0.296</td>
<td>0.130</td>
<td>0.158</td>
</tr>
</tbody>
</table>

The qualitative change, expressed in enhanced lipid fluidity and rotational freedom of the protein, was accompanied by a 10-fold reduction in ADA activity (11). The same phenomenon repeated in RSV-Ts68-infected cells grown at 36 °C (at which they are transformed); their profile (Fig. 3B) approached that of RSV-transformed cells. The control obtained by growing these cells at 41 °C (at which they resumed "normal" properties) produced the normal double-humped profile (Fig. 3B) of high phase values. There is a correspondence between the profiles and the associated ADA activities, only 4-5-fold reduction in ADA activity in Ts-68-transformed cells grown at 36 °C versus a 10-fold reduction in the RSV-transformed CEF (11). The same trends were discerned at a single (6 MHz) frequency on the SLM 4800 spectrophotofluorimeter.3

Table I shows steady state fluorescence polarization data with three lipophilic probes (23, 24). These data indicate increased microfluidity of the lipid core, associated with cell transformation (RSV-Ts68 at 36 °C), which returns to normal when the RSV-Ts68-infected CEF are grown at 41 °C (25). Lifetimes of DPH and 10-(1-pyrene)-10-ketodecanoic acid measured by single photon correlation (4) and phase modulation (22) fluorimetry showed no change, thus supporting the assignment of polarization changes to microfluidity.

Our results with lipophilic probes associate CEF transformation with a fluidized lipid core of the membrane. The large ADA complex, a membrane protein, indicates faster segmental rotational diffusion, presumably allowed by the more fluid environment. A word of caution is noteworthy; the observed reduction in pyrene-SS-ADA lifetime (or in the fractional contribution of the long lifetime component) (Fig. 3) in the transformed cells should have raised the profile (Fig. 3B). Yet, the eventual collapse of this profile indicates the dominant role of faster rotational relaxation of the protein. The shortened lifetime (observed in transformed cells) may originate in diffusion-limited quenching, expected to increase in the more fluid membrane.

The qualitative change, expressed in enhanced lipid fluidity and rotational freedom of the protein, was accompanied by a quantitative change; transformed cells attracted less of the externally applied SS-ADA as compared with the normal or "revertant" cells (Fig. 2b). (Careful analysis (26) of fluorescence lifetimes supported this conclusion.) One possible explanation of the lowered binding is that less ADCP is synthesized in transformed CEF. We would suggest the additional possibility that ADCP present in the membrane is less available to pyrene-SS-ADA binding because of conformational
protein is affected by the modified lipids. The possibility that outwardly exposed ADCP is less abundant on the transformed cell surface was suggested before (5). The reduced binding of SS-ADA to the sunken ADCP combined with the reduced availability of the large ADA complex to the hydrophilic substrate, adenosine, may account in part for the observed reduction in ADA activity in transformed cells. We thus conclude that ADCP has a basic regulatory role in ADA activity. Work is in progress on the interesting problem as to whether ADCP is nearly absent or is present but inactive in transformed cells. Analogous problems are encountered in the biochemistry of aging.

Cell detachment, exclusive to normal cells, is related to extensive ADA binding to the abundant membranal ADCP. A plausible explanation of this unexpected phenomenon is that the normal fibroblast, densely coated with hybrid ADA, fails to adhere to the culture flask.

In conclusion, spectroscopic evidence has been presented on the role of membrane dynamics in modifying the activity of a membrane-bound enzyme in transformed cells.

A preliminary report of this work was presented at the 32nd Annual Meeting of the American Biophysical Society, Phoenix, AZ, February 28, 1988 (27).

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REFERENCES


