An ascamycin dealanylating enzyme (Xc-aminopeptidase) has been isolated from Xanthomonas citri and characterized as a proline iminopeptidase of molecular weight of 38,000 (H. Osada & K. Isono, Biochem. J. 233: 459–463, 1986). Immunological studies have shown that all the mammalian cells tested possess a closely-related enzyme(s) on their cell membranes. This enzyme is more active in transformed cells than in nontransformed cells. A decreased ratio of ID₅₀ (ascamycin/dealanylascamycin based on [³⁵S]methionine uptake) in transformed cells compared with the nontransformed cell can be explained on the basis of the conversion of ascamycin to dealanylascamycin by the enzyme. It is suggested that ascamycin cannot be transported through mammalian cell membranes but dealanylascamycin can permeate; a similar situation exists in prokaryotic cells.

Ascamycin is an unique nucleoside antibiotic having an alanyl-sulfamoyl group on the 5'-oxygen of 2-chloroadenosine. It has selective antibacterial activity against plant pathogenic Xanthomonas species, while the dealanylated derivative shows broad antibacterial activity. We have shown that both ascamycin and dealanylascamycin inhibit bacterial protein synthesis in a cell free system but the antibacterial activity of ascamycin is masked by the alanyl group which interferes with transport into bacteria. Xanthomonas citri is susceptible to ascamycin by virtue of an ascamycin dealanylating enzyme which exists on the cell surface (Fig. 1). Other bacteria which lack the enzyme are not susceptible to ascamycin. We have isolated and characterized the ascamycin hydrolyzing aminopeptidase from X. citri. The enzyme, molecular weight of 38,000, can be classified as a proline iminopeptidase [EC.3.4.11.5].

The purpose of this study was to examine the distribution of the ascamycin dealanylating enzyme in mammalian cells. Our experiments have shown that transformed cells possess generally higher ascamycin dealanylating activity than nontransformed cells.

Materials and Methods

Cell Lines and Culture Conditions

The cell lines used are listed in Table 1. Mouse derived cell lines, Balb3T3 clone A31, SV40 virus transformed cell SV-3T3, and Kirsten sarcoma virus transformed cell Ki-3T3 were grown in Eagle’s minimal essential medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (Gibco). Rat derived cell lines (NRK and its Harvey sarcoma virus transformed cell) and mouse derived cell lines (NIH L1 and its chemical carcinogen transformant L1-4NQO) were cultured under the same conditions as Balb3T3 cell lines. Human chronic myeloid cell, K-562, was grown in RPMI 1640 medium (Gibco) containing 10% serum and antibiotics (penicillin and streptomycin). These cell lines were gifts from Dr. K. Kohno (National Institute for Basic Biology, Okazaki). Human promyeloid leukemia cell, HL60, was cultured under the same conditions as K-562. Friend mouse
erythroleukemic cells\textsuperscript{12) were grown in EAGLE’s minimal essential medium containing 12.5\% fetal bovine serum. HL60 and Friend cells were provided by Dr. S. Nomura (University of Tokyo).

Cells were incubated in a humidified atmosphere (5\% CO\textsubscript{2}) at 37°C. Cell morphology was observed by phase contrast microscopy (TMD, Nikon). Doubling time were calculated from the growth curve of the control culture.

**Preparation of Subcellular Fractions**

Adherent cells were harvested with a rubber policeman without trypsin. Floating cells were harvested by centrifugation. Both types of cells were washed with phosphate buffered saline, PBS(—), and disrupted by a sonicator (UR200, Tomy Seiko) under mild conditions to preserve intact nuclei (150W, 20 seconds). After the cell lysate was adjusted to a sucrose concentration of 8.6\%, it was overlaid onto 37\% sucrose in 10 mm Tris-HCl (pH 7.8) in a centrifuge tube.

After centrifugation at 65,000 \times g for 60 minutes at 4°C in a swinging rotor (RPS-40T, Hitachi), the upper layer contained cytoplasmic proteins or soluble proteins, the boundary fluff was a membrane rich fraction, and the pellet contained nuclei and unbroken cells. The protein concentration of each subcellular fraction was adjusted to 3 mg/ml, stored at −20°C, and used for assays.

**Assay for Proline Iminopeptidase Activity**

Each subcellular fraction was used at a concentration of 0.1 mg protein per ml in the reaction mixture. Spectrophotometric measurement was carried as previously described\textsuperscript{3}).

**Preparation of Antiserum against Xc-aminopeptidase**

The antiserum was obtained by two subcutaneous injections of 1 mg of Xc-aminopeptidase at 10-day intervals to two rabbits (New Zealand white, female, 2 kg). The Xc-aminopeptidase in 0.5 ml
of Tris-HCl buffer was emulsified with 0.5 ml of Freund's complete adjuvant for the first injection and
emulsified with 0.5 ml of Freund's incomplete adjuvant for the second injection. The antiserum was
collected from the rabbit 10 days after the second injection. The titer of anti-Xc-aminopeptidase was
estimated by the OUCHTERLONEY method\textsuperscript{(13)}. Normal serum did not react with Xc-aminopeptidase
but immune serum diluted 1/128 did cross-react. The serum was partially purified by 30% ammonium
sulfate precipitation, which was used as the anti-Xc-aminopeptidase IgG rich fraction.

Indirect Immunofluorescence Technique

Adherent cells were cultured to preconfluence in the Lab-Tek chamber (Miles, Naperville, Illinois).
In the case of floating cells, cultured cells were harvested by centrifugation and spread onto a glass slide.
Both types of cells on the slide were washed with two changes of PBS(–) and fixed with 3.7% formalin
in PBS(–) for 20 minutes. Then the cells were washed with PBS(–) and incubated with 30-fold
diluted anti-Xc-aminopeptidase IgG rich fraction in PBS(–) for 45 minutes at 37°C. After the cells
were washed, they were incubated for 30 minutes at room temperature with FITC (fluorescein isothio-
cyanate)-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Maryland) at a
concentration of 10 μg/ml. Well washed samples were observed by fluorescence microscopy (XF-
EFD, Nikon).

Western Blotting

Each 50 μg sample of cell membrane protein was applied to sodium dodecyl sulfate (SDS)-poly-
acrylamide gel (12.5%) electrophoresis\textsuperscript{(14)}. The developed proteins were transferred to a nitrocellulose
membrane using the Bio-Rad electro-blotting system. Following binding of the antigen to the mem-
brane, the remaining unbound sites were blocked with gelatin. The membrane with bound antigen
was incubated with the first antibody (anti-Xc-aminopeptidase IgG), washed to remove unbound
antibody, incubated with the second antibody (horse radish peroxidase-conjugated goat anti-rabbit
IgG), and washed again. 4-Chloro-l-naphthol was used as substrate for horse radish peroxidase and
the developed membrane showed purple bands. All reagents used in this procedure were purchased
from Bio-Rad (Richmond, CA).

Susceptibility of Cells to Ascamycin and Dealanylascamycin

Since both ascamycin and dealanylascamycin inhibit protein synthesis, inhibition of [\textsuperscript{35}S]methionine
uptake into the acid-insoluble fraction was measured in the presence of antibiotic. Cells were grown
in each medium to the logarithmic phase, then a series of antibiotic dilution was added. After 20
hours incubation, the medium was changed to fresh Ham's F-12\textsuperscript{(15)} containing 10% serum, each con-
centration of antibiotic, and 5 μCi/ml [\textsuperscript{35}S]methionine (1,120 Ci/mmol, New England Nuclear). The
cells were labeled for 3 hours, washed, and harvested. After treatment with ice-cold trichloroacetic
acid, radioactivity in the acid-insoluble fraction was measured by a liquid scintillation counter.

Results

Proline Iminopeptidase Activity of Normal and Transformed Cells

Proline iminopeptidase activity of the cell line subcellular fractions listed in Table 1 was measured.
In the course of purification of Xc-aminopeptidase from X. citri, we had observed that proline
iminopeptidase activity was proportional to ascamycin dealanylating activity\textsuperscript{(3)}. In each types of
cells, the activity was localized on the cell membrane and the activity of transformed cells was higher
than that of the nontransformed cells (Table 1).

The dealanylation of ascamycin can be detected semi-quantitatively by the decrease of Rf value on
cellulose thin-layer chromatography and the appearance of antibacterial activity against Escherichia
coli BE1186\textsuperscript{(3)}. The membrane fraction of SV-3T3 showed higher dealanylation activity than that of
Balb3T3 (Fig. 2). Because of the limited supply of ascamycin, ascamycin dealanylating activity of
the other cells was not examined.
The presence of antigen to anti-Xc-aminopeptidase IgG on the mammalian cell membranes was demonstrated in the following two ways.

1) Indirect Immunofluorescence Microscopy: Intense fluorescence was observed on the cell membranes of transformed cells while only slight fluorescence was observed on those of nontransformed cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transforming agent</th>
<th>Doubling time (hours)</th>
<th>Anchorage independent growth</th>
<th>Proline iminopeptidase activity&lt;br&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Balb3T3</td>
<td></td>
<td>13.7</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>SV-3T3</td>
<td>SV40</td>
<td>13.3</td>
<td>+</td>
<td>0.6</td>
</tr>
<tr>
<td>Ki-3T3</td>
<td>Kirsten-MSV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.5</td>
<td>+</td>
<td>3.6</td>
</tr>
<tr>
<td>NRK</td>
<td></td>
<td>24.0</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Ha-NRK</td>
<td>Harvey-MSV</td>
<td>15.1</td>
<td>+</td>
<td>2.0</td>
</tr>
<tr>
<td>NIH L1</td>
<td>4-Nitroquinoline-1-oxide</td>
<td>17.5</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>K-562</td>
<td>(Human leukemia)</td>
<td>13.1</td>
<td>(+)</td>
<td>3.3</td>
</tr>
<tr>
<td>HL60</td>
<td>(Human leukemia)</td>
<td>19.6</td>
<td>(+)</td>
<td>0.8</td>
</tr>
<tr>
<td>Friend</td>
<td>Friend virus</td>
<td>10.0</td>
<td>(+)</td>
<td>1.5</td>
</tr>
<tr>
<td>L1-4NQO&lt;sub&gt;4d&lt;/sub&gt;</td>
<td>4-Nitroquinoline-1-oxide</td>
<td>11.4</td>
<td>+</td>
<td>1.8</td>
</tr>
</tbody>
</table>
| Fig. 2. Dealanylation of ascamycin by cell fractions as detected by cellulose thin-layer chromatography [solvent system, 2-propanol - 1 N ammonia (7: 3)].
  a, Ascamycin; b, dealanylascamycin; c~i, reaction products of ascamycin treated subcellular fraction; c, Xc-aminopeptidase from X. citri; d~f, subcellular fractions of Balb3T3; g~i, subcellular fractions of SV40 transformed Balb3T3; d, g, cytoplasmic proteins; e, h, membrane bound proteins; f, i, nucleus and unbroken cells.

Table 1. Characteristics of cell lines and their proline iminopeptidase activity.<sup>a</sup>

<sup>a</sup> Enzyme activity (nmol/minute/mg protein) was assayed as described<sup>7</sup> using L-proline-β-naphthylamide as substrate.

<sup>b</sup> Anchorage independent growth was indicated by the colony-forming ability in semi-solid medium<sup>16</sup>.

<sup>c</sup> Crude enzymes were prepared by sucrose density gradient centrifugation as described in the text. Precipitate contained nucleus and unbroken cells.

<sup>d</sup> Murine sarcoma virus.

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**Presence of Enzyme(s) Similar to Xc-aminopeptidase**

The presence of antigen to anti-Xc-aminopeptidase IgG on the mammalian cell membranes was demonstrated in the following two ways.

1) Indirect Immunofluorescence Microscopy: Intense fluorescence was observed on the cell membranes of transformed cells while only slight fluorescence was observed on those of nontransformed cells.

**Fig. 2.**

**Fig. 3.** Indirect immunofluorescence microscopy.

a, Balb3T3; b, SV-3T3; c, Ki-3T3; d, K-562; e, HL60; f, Friend cell.

a~c, Observed at a magnifying ×200; d~f, ×100.
cell (Figs. 3a–c). Fluorescence of transformed cells was stronger than that of the nontransformed cell with other cell lines (data not shown). FITC-positive material was detected not only on murine cells but also on human cells (Figs. 3d, 3e).

2) Western Blotting: All the cell membranes tested were found to have antigen cross-reacting with anti-Xc-aminopeptidase IgG (Fig. 4). The bands appearing on the nitrocellulose membrane indicated that they had the almost same molecular weight as Xc-aminopeptidase (38,000 daltons). Virus transformed cells, especially Ki-3T3 and Ha-NRK contained a large amount of the antigen. It is to be noted that both cell lines possess the ras oncogene whose product is a 21,000-dalton protein\(^{7,16}\).

Susceptibility of Normal and Transformed Cells to Ascamycin and Dealanylascamycin

Many bacteria other than Xanthomonas species and fungi are resistant to ascamycin (i.e. they tolerate 200 \(\mu M\) of ascamycin). However all the mammalian cells tested were susceptible to ascamycin at ID\(_{50}\) (50\% inhibition dose) values of 0.5 to 6.6 \(\mu M\) (Fig. 5).

As described previously in the case of prokaryotic cells, ascamycin is considered not to be able to permeate the membrane while dealanylascamycin is transported into the cytoplasm\(^{5}\). If this is also the case for eukaryotic cells, the ratio of ID\(_{50}\) of ascamycin and dealanylascamycin (Table 2) may represent an inverse index of the conversion efficiency from ascamycin to dealanylascamycin, hence the efficiency of membrane transport of ascamycin.

The proline iminopeptidase activity (shown in Table 1) was plotted in Fig. 6 against the ratio of ID\(_{50}\) of ascamycin to dealanylascamycin (shown in Table 2). Statistical calculation indicated that there is the strong correlation between two factors. These data suggest that proline iminopeptidase activity is increased in transformed cells, resulting in the increase of susceptibility to ascamycin. It is considered that ascamycin is dealanylated by the enzyme, and then transported across the membrane.

Discussion

We have already reported the mode of action and the selective toxicity of ascamycin in bacteria\(^2\).
In the present paper, we have shown that an ascamycin dealanylating enzyme is also present in mammalian cell membranes and the activity is higher in transformed cells than in the non-transformed cell. The enzyme activity was associated with proline iminopeptidase which was closely related to ascamycin dealanylating enzyme, Xc-aminopeptidase. An immunological technique revealed that all cells tested contained the immunologically related Xc-aminopeptidase and that this antigen increased in the transformed cells compared with the non-transformed cells.

Aoyagi et al.,17,18) measured activities of various hydrolyzing enzymes in rat organ homogenates and on the cell surface of cells from various sources, i.e., tumor cell lines, primary cultured cells, normal cell lines, and their transformants. They revealed that the proline iminopeptidase activity of FM3A cells, mouse mammary tumor cells, was higher than that of normal cells. The result agrees with our observation in this paper, but further investigation is necessary to reveal the relationship between cell

![Fig. 5. Inhibition curve of ascamycin and dealanylascamycin.](Image)

![Fig. 6. Correlation between proline iminopeptidase and susceptibility ratio (ID<sub>50</sub> of ascamycin/dealanylascamycin).](Image)

![Table 2. Effects of ascamycin and dealanylascamycin on protein synthesis.](Table)

<table>
<thead>
<tr>
<th>Cell</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (10&lt;sup&gt;-6&lt;/sup&gt; M) Ascamycin</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (10&lt;sup&gt;-9&lt;/sup&gt; M) Dealanylascamycin</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb3T3</td>
<td>1.4</td>
<td>1.9</td>
<td>730</td>
</tr>
<tr>
<td>SV-3T3</td>
<td>3.8</td>
<td>11</td>
<td>350</td>
</tr>
<tr>
<td>Ki-3T3</td>
<td>2.5</td>
<td>21</td>
<td>120</td>
</tr>
<tr>
<td>NRK</td>
<td>2.2</td>
<td>4.5</td>
<td>490</td>
</tr>
<tr>
<td>Ha-NRK</td>
<td>4.0</td>
<td>25</td>
<td>160</td>
</tr>
<tr>
<td>NIH L1</td>
<td>1.5</td>
<td>4.1</td>
<td>370</td>
</tr>
<tr>
<td>L1-4NQQ&lt;sub&gt;ad&lt;/sub&gt;</td>
<td>1.1</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>K-562</td>
<td>0.5</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>HL60</td>
<td>3.0</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>Friend</td>
<td>6.6</td>
<td>43</td>
<td>150</td>
</tr>
</tbody>
</table>

<sup>a</sup> ID<sub>50</sub> of ascamycin/ID<sub>50</sub> of dealanylascamycin.
transformation and the increase of proline iminopeptidase activity observed in this paper.

In bacteria, ascamycin cannot be transported into the cytoplasm because the alanyl group of ascamycin interferes with membrane transport; dealanylascamycin is transportable. If this is also the case with mammalian cells, the ratio of 50% inhibition dose of methionine uptake between ascamycin and dealanylascamycin can be regarded an inverse index of dealanylation of ascamycin, i.e., membrane transport of ascamycin.

We have shown that there exists a clear relationship between proline iminopeptidase activity and ascamycin susceptibility in mammalian cells. In this respect, a prolyl analog of ascamycin might be worthwhile for investigation. We have already succeeded in total synthesis of ascamycin and the synthesis of the prolyl analog is in progress.

Acknowledgments

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References

