Identification of a Cold-sensitive Step in the Mechanism of Modeccin Action

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Modeccin is a toxic lectin that arrests protein synthesis in mammalian cells by catalytically inactivating 60 S ribosomes. To interact with 60 S ribosomes, the catalytic subunit of modeccin must pass through a membrane and enter the cytosol. Two known steps in the mechanism of modeccin action are the receptor-mediated internalization of the toxin into vesicles and a second step that requires a low pH within the vesicles. We report here another step required for modeccin to arrest protein synthesis, identified because this step was blocked at 15 °C. Modeccin traveling from cell surface receptors to the cytosol at 37 °C passed the low pH step within vesicles in a minimum time of 15 min after endocytosis and reached the cold-sensitive step 15 min later. There was no effect on protein synthesis until about 45 min after modeccin had passed the cold-sensitive step, suggesting that the toxin was still within vesicles at the time of the cold-sensitive event. The low temperature at which modeccin failed to reach the cytosol correlated with an apparent low temperature block in the transfer of endocytosed modeccin to lysosomes. The possibility is discussed that modeccin does not penetrate to the cytosol directly from endocytic vesicles.

Many viruses and protein toxins damage mammalian cells only after maneuvering from cell surface receptors, through a membrane barrier, and into the cytosol. The best known route to the cytosol is that of Semliki Forest virus described by Marsh et al. (1). After binding to surface receptors, viral-receptor complexes are internalized into the acidic endosomal compartment where the low pH induces fusion of the viral membrane with the endosomal membrane, passing the nucleocapsid through to the cytosol. Diphtheria toxin, a protein that catalytically arrests protein synthesis in target cells, follows a similar invasive route except that the low endosomal compartment where the low pH induces fusion of the viral membrane with the endosomal membrane, passing the nucleocapsid through to the cytosol. Diphtheria toxin is bound to cell surface receptors and the cells are exposed to medium at a low pH of 5 or below, the A fragment of the toxin translocates directly through the plasma membrane into the cytosol (2-8). This suggests that the cell supplies at least one other function, in addition to a low pH, that acts on modeccin within the vacuolar compartment to allow membrane penetration.

When incubated with cells at temperatures between 20 and 15 °C, both diphtheria toxin (1,2) and Semliki Forest virus (1) are endocytosed, find a low pH, and penetrate to the cytosol; however, neither agent reaches lysosomes at low temperature, indicating that they pass to the cytosol through a prelysosomal membrane. We have characterized here the effect of low temperature on the destination of modeccin. At 15 °C, modeccin was endocytosed and encountered a low pH, but the toxin apparently reached neither the cytosol nor the lysosomes. Moreover, after modeccin was endocytosed at 37 °C, a minimum time of about 75 min elapsed before the toxin first had an effect on protein synthesis. These results are consistent with the possibility that modeccin does not pass into the cytosol from early endosomal vacuoles.

EXPERIMENTAL PROCEDURES

Materials—Modeccin and Iodobeads were purchased from Pierce Chemical Co. Abrin was from Sigma. Rabbit anti-modeccin was raised against formaldehyde-treated modeccin as described by Kebir et al. (10). L-[4,5-3H]Leucine (60 Ci/mM) was from Schwarz-Mann. Carrier-free Na[125I] was purchased from Amersham-Searle Corp. Bio-Gel P-10 was from Bio-Rad. Vero cells (ATCC registry number CCL81), used in all experiments, were from the American Type Culture Collection, Rockville, MD. Sources of other chemicals and reagents are indicated in the text.

supplies, all from routine vendors, have been previously identified (5).

Methods—Vero cells were routinely grown in an atmosphere of 10% CO₂, 90% air with Dulbecco's modified Eagle's medium containing 5% fetal bovine serum as described previously (5). Many of the experimental incubations were done in ambient air, in which case the medium contained no bicarbonate and was buffered with 20 mM HEPES. The effect of modeccin on protein synthesis was assayed by the incorporation of [³H]leucine into trichloroacetic acid-insoluble material as detailed in an earlier report (5). When incorporation was measured above 20 °C, the medium, hereafter called medium A, contained leucine reduced by 100-fold (8 μM). At 15 °C, the rate of protein synthesis in Vero cells was about 10% that at 37 °C. To enhance incorporation of [³H]leucine at low temperature and during short incubation periods at 37 °C, we used medium containing no endogenous leucine, called medium B. [³H]Leucine was added as described in the figure legends. Anti-modeccin was present at 5% (v/v) in medium. Our preparation of anti-modeccin completely neutralized the cytotoxic activity of 10⁻⁶ M modeccin when the antisera was added to cells before the modeccin. When modeccin was prebound to cells at 4 °C, however, the protection by the antisera was incomplete at high toxin concentrations, suggesting that the anti-modeccin did not effectively remove toxin from the cell surface. Protection was complete at all toxin concentrations when cells with modeccin on their surface were incubated at 4 °C for 1 h with anti-modeccin and 0.1 M lactose. Lactose, which binds to modeccin at the receptor-binding site, prevents interaction of the toxin with receptors and enhances removal from the cell surface (8, 11).

Modeccin was prepared with iodobeads according to the manufacturer's instructions. One iodobead was washed twice with buffered saline, blotted dry on filter paper, and added to 40 μl of modeccin (1 mg/ml) in buffered saline. Iodination was initiated by addition of NaI (1 M) (1 nCi). After 16 min, the reaction was stopped by removing the solution from the bead. 50 μl of KI (40 mg/ml in buffered saline) were added, and the mixture was chromatographed on a column of Bio-Gel P-10 equilibrated with buffered saline and 1 mg/ml of bovine serum albumin to separate modeccin from free iodide. Modeccin was recovered with a yield of nearly 100% and a specific activity of 6 × 10⁶ cpm/μg.

Cells were incubated with 10⁻⁸ M ¹²⁵I-modeccin as described in the legend to Fig. 3. Acid-soluble radioactivity in the medium was measured by mixing 0.5 ml of medium with an equal volume of cold 10% (w/v in water) trichloroacetic acid in a microfuge tube. After 20 min, the mixture was centrifuged for 5 min and the radioactivity in the supernatant was assayed with a Beckman model 5500 γ counter. Galactose, which inhibits binding of modeccin to cells (8, 11), was included in the medium of controls to assess the acid-soluble radioactivity in the medium derived from nonspecifically internalized ¹²⁵I-modeccin. Cell-associated radioactivity was assayed after cells were dissolved in 1.0 N NaOH.

RESULTS

Cytocidal Activity at Low Temperature—Vero cells were exposed to different concentrations of modeccin at the temperatures shown in Fig. 1 and assayed for protein synthesis 24 h later. At about 20 °C and below, the concentration of modeccin required to inhibit protein synthesis increased dramatically until at 15 °C there was no effect, even at 100 nM toxin. For comparison, the toxic lectin abrin, which inactivates 60 S ribosomes as does modeccin (9), still arrested protein synthesis at 15 °C (Fig. 1, lower right). Diphtheria toxin1 and Pseudomonas aeruginosa exotoxin A² also retain cytocidal activity at 15 °C.

The effect of low temperature on modeccin could be due to a block in the receptor-mediated endocytosis of the toxin. To test this, cells were incubated with different concentrations of modeccin at 15 °C for 3 h and treated with antitoxin and lactose at 4 °C to neutralize any accessible toxin. Protein synthesis was assayed after 5 h at 37 °C to detect the activity of any toxin that had been sequestered within vesicles during

1 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 M. Stookey and R. K. Draper, unpublished data.

![FIG. 1. The effect of low temperature on the cytotoxic activity of modeccin. Cells were exposed to modeccin at the indicated temperatures for a total of 24 h. Above 20 °C, protein synthesis was measured after cells were incubated for 1 h in medium A containing 2 μCi/ml of [³H]leucine. At 20 °C and below, protein synthesis was measured after cells were incubated for 2 h in medium B containing 10 μCi/ml of [³H]leucine. Protein synthesis is expressed relative to controls that received no toxin. △, 37 °C; O, 30 °C; □, 20 °C; △, 18 °C; ▲, 15 °C; Δ, cells treated with 10 nM abrin at 15 °C as above. The concentration of abrin that inhibited protein synthesis by 50% at 37 °C was 3 × 10⁻¹⁵ M.]

![FIG. 2. Modeccin becomes refractory at 15 °C to inhibition by external anti-modeccin and lactose. Cells were incubated at 15 °C for 3 h with the indicated concentrations of modeccin, treated with anti-modeccin and 0.1 M lactose at 4 °C for 1 h, washed, and incubated for a total of 5 h at 37 °C. During the last hour, 2 μCi/ml of [³H]leucine were present in medium A. To show that the anti-modeccin and lactose neutralized toxin on the cell surface, the closed square in the upper right indicates protein synthesis when cells were preincubated for 1 h at 4 °C with 100 nM modeccin, treated with anti-modeccin and lactose for 1 h at 4 °C, and then placed at 37 °C for 5 h. The open square at the lower right indicates protein synthesis when the anti-modeccin and lactose were omitted in this control. Protein synthesis is relative to cells that received no toxin.](http://www.jbc.org/article/S0021-9525(17)30580-9/abstract)
at various times after addition of toxin at 15 °C, the cells were treated with antitoxin and lactose at 4 °C for 1 h and placed at 37 °C for 6 h before measuring protein synthesis. Within 25 min at 15 °C, greater than 90% of the cells had internalized sufficient modeccin to arrest protein synthesis (data not shown).

**Time Required to Pass Inhibitor-sensitive Steps**—Three of the steps that modeccin must negotiate in order to arrest protein synthesis can be marked with appropriate inhibitors. 1) Insensitivity to inhibition by antitoxin and lactose in the extracellular medium indicates endocytosis; 2) insensitivity to ammonium chloride presumably identifies an acidification step within vesicles; and 3) low temperature marks an unknown step. To determine the minimum times required for modeccin to pass each of these steps, cells were preincubated at 4 °C with excess toxin to saturate surface receptors, placed at 37 °C, and then exposed after different times to an inhibitory condition. Protein synthesis was assayed 6 h later at 37 °C or 24 h later at 15 °C to determine that fraction of the cells in which modeccin had passed a given inhibitor-sensitive point. Control experiments indicated that the major effect of the toxin on protein synthesis was complete after these times. The data are presented in a semilog plot in Fig. 3. The fraction of intoxicated cells passing the inhibitor-sensitive steps increased as a first order function of time, reflecting the random probability for a given cell to internalize a lethal dose of the toxin (12). The curves were extrapolated to the time of 100% protein synthesis to estimate the interval before modeccin first passed each step affected by an inhibiting condition. Insensitivity to antitoxin and lactose, indicating endocytosis, began immediately upon warming the cells, while insensitivity to ammonium chloride and low temperature first arose 15 and 30 min after endocytosis, respectively. This suggested that the step affected by low temperature occurred after the toxin was endocytosed and exposed to a low pH within vesicles.

To measure the actual rate of protein synthesis in the cells as modeccin was passing the steps affected by the different inhibitors, cells were preincubated with 100 nM modeccin at 4 °C, placed at 37 °C, and assayed for protein synthesis after different times. As shown in Fig. 3, no decline in protein synthesis occurred until about 75 min after the toxin was endocytosed. This suggested that the A chain of modeccin was still within intracellular vesicles at the time of the cold-sensitive step.

The times shown in Fig. 3 are minimal times since increasing the toxin concentration did not shorten the intervals. Moreover, adding modeccin back to the culture medium after the cells were placed in medium at 37 °C did not alter the results, indicating that any dissociation of the toxin from receptors into the medium did not reduce the amount of toxin on the cell surface to an extent sufficient to lengthen the intervals.

**Inhibitor Exchange Studies**—That the amine-sensitive step occurred before the cold-sensitive step was verified by inhibitor exchange experiments, an approach previously described by Marnell et al. (5) and Marsh et al. (13). If modeccin will pass the amine-sensitive step when incubated with cells at 15 °C, then toxin that has passed the amine-sensitive point may proceed to the cytosol and arrest protein synthesis when the inhibiting conditions are exchanged, that is, when ammonium chloride is added and the cells are placed at 37 °C to release the low temperature block. The results of one set of experiments designed to test this prediction are shown in Table I. Experiment 1 was a control to establish that the

![Fig. 3. Kinetics of modeccin import.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Protein synthesis</th>
<th>Radioactivity</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 °C (24 h)</td>
<td>37 °C (75 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl Modeccin NH₄Cl</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>− − −</td>
<td>1.5 10²² 130</td>
<td>0.2 12</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+ − +</td>
<td>1.3 10⁰ 100</td>
<td>1.2 92</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>− + +</td>
<td>1.5 10⁰ 100</td>
<td>0.4 27</td>
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inhibition observed at low temperature was reversible. Cells were incubated with modeccin at 15 °C for 24 h and then placed at 37 °C to measure protein synthesis. Protein synthesis was arrested, demonstrating reversibility. Experiment 2 was another control to determine whether the ammonium chloride block could be established at 15 °C. Cells at 15 °C were treated with ammonium chloride and exposed at this temperature to toxin for 24 h. The temperature was elevated to 37 °C in the presence of ammonium chloride, and protein synthesis was assayed 1 h later. The effect of the toxin on protein synthesis was inhibited, suggesting that the ammonium chloride block was established at low temperature. In Experiment 3, cells were exposed to modeccin for 24 h at 15 °C, treated with ammonium chloride, and then placed at 37 °C in the presence of ammonium chloride. Protein synthesis was inhibited, indicating that modeccin had passed the amine-sensitive step at 15 °C so that this step must have occurred before the cold-sensitive step.

In another experiment, cells were incubated for different times at 15 °C with 100 nM modeccin, treated with ammonium chloride, and then incubated at 37 °C before measuring protein synthesis to determine the time required to pass the acidification step at low temperature. Four hours elapsed before the toxin first passed the amine-sensitive step, and the maximum effect developed after 12 h at 15 °C (data not shown). We conclude that modeccin does pass the amine-sensitive step, although slowly, at 15 °C.

We also did the reciprocal inhibitor exchange experiments whereby cells were first exposed to ammonium chloride and modeccin at 37 °C and then incubated at 15 °C after the amine was removed. In a control experiment to establish that the effects of ammonium chloride were reversible, cells were incubated with 100 nM modeccin at 37 °C for 1 h in the presence of ammonium chloride, washed three times with medium to remove the drug, and assayed for protein synthesis 2 h later. We used three thorough washes to remove the drug because Sandvig et al. (14) had previously reported difficulty in reversing the inhibitory effect of ammonium chloride on the action of modeccin. Protein synthesis was 10% of normal, indicating that the toxin inhibitory effect of the drug was reversed. If the ammonium chloride was not removed, modeccin did not arrest protein synthesis. To again test whether the amine-sensitive step occurred before the cold-sensitive step, cells were incubated with 100 nM modeccin and ammonium chloride at 37 °C for 1 h, chilled to 15 °C, and washed to remove the ammonium chloride. Protein synthesis was assayed after 24 h at 15 °C. Protein synthesis was 90% of normal, as predicted if the toxin had not passed the cold-sensitive point in the presence of ammonium chloride (data not shown).

Effect of Low Temperature on the Lysosomal Digestion of Modeccin—To see if modeccin was degraded within lysosomes at 15 °C, cells were incubated 4 h at 15 °C with radiolabeled toxin, chilled, washed with galactose to remove toxin remaining on the cell surface, and placed at either 15 or 37 °C, and the acid-soluble radioactivity appearing in the medium was measured. As seen in Fig. 4, modeccin previously internalized at 15 °C rapidly appeared in the medium as acid-soluble fragments at 37 °C, while no acid-soluble material appeared at 15 °C. The appearance of acid-soluble radioactivity as a function of temperature is shown in the inset of Fig. 4; there was little excretion of degraded toxin below about 20 °C.

Dunn et al. (15) have shown that asialofetuin is not transferred from endosomes to lysosomes at 16 °C in rat hepatocytes. Marsh et al. (1) reached a similar conclusion regarding the destination of Semliki Forest virus within BHK-21 cells at low temperature. In both of these studies, lysosomal extracts retained at least partial proteolytic activity at low temperature; thus, if endocytosed material did reach lysosomes at low temperature, it should be susceptible to degradation. Our observation that modeccin was not degraded at 15 °C, even after 4 h, suggests that modeccin did not reach lysosomes at the low temperature. Evidence of Sandvig and Olsnes (16) also indicates that abrin and ricin are not degraded by cells below about 20 °C.

**DISCUSSION**

Low temperature might interfere with any of three main events required for modeccin to arrest protein synthesis: 1) the process of receptor-mediated endocytosis, either at the toxin-receptor interaction step or the step where occupied receptors are internalized; 2) the transfer of endocytosed modeccin from within vesicles to the cytosol; and 3) the inactivation of 60 S ribosomes by the A chain in the cytosol. The first possibility is unlikely. Radiolabeled modeccin incubated with cells at 15 °C became resistant to release from the cell surface by external galactose, suggesting that the toxin had bound to receptors and was sequestered within vesicles. Furthermore, modeccin that was incubated with cells at 15 °C inhibited protein synthesis after the cells were treated to neutralize toxin remaining on the cell surface and placed at 37 °C, suggesting that receptor-bound toxin was transferred.
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to intracellular vesicles at the low temperature. Similar results were previously reported for abrin and ricin (12, 16). The third possibility, that the A chain of modeccin reaches the cytosol only to be ineffective at the low temperature, is also unlikely. About 45 min elapsed before a decline in protein synthesis after the toxin had passed the cold-sensitive point at 37 °C. If the A chain was free in the cytosol at the cold-sensitive point, an effect on protein synthesis should have occurred without a delay of 45 min. Moreover, the A chains of abrin and ricin, both of which modify 60 S ribosomes as does modeccin, retain modification activity in cell-free systems at 15 °C (17). Abrin also retained cytotoxic activity at 15 °C (Fig. 1). This suggests that low temperature per se should not protect ribosomes from modification by the A chain of modeccin were the A chain to reach the cytosol at 15 °C. Although not conclusive, the evidence is most compatible with the idea that modeccin enters the vacuolar compartment at 15 °C but is not transferred to the cytosol.

The kinetics of import indicates that modeccin began to pass the amine-sensitive point, presumably acidification inside vesicles, within 15 min of endocytosis and 15 min later began to pass the cold-sensitive point. The order in which these steps occurred was confirmed by inhibitor exchange studies. There is some uncertainty in the time required before the toxin passed the cold-sensitive point since we do not know the time required to establish the low temperature block after cells were exposed to medium at 15 °C. Thus, the cold-sensitive point could be at some time earlier than 15 min after the acidification step. A similar uncertainty should not apply to amine inhibition because ammonium chloride elevates the pH inside acid intracellular vesicles within 60 s of drug addition (18).

Two lines of evidence suggest that modeccin does not pass to the cytosol directly from an early endosomal compartment. First, the A chain apparently did not reach the cytosol until around 75 min after endocytosis at 37 °C. The rapid migration of endocytosed membrane to other sites is well documented (19), and it seems unlikely that modeccin would sit within the same compartment for 75 min. If modeccin molecules en route to the cytosol do not move out of endosomes during this time, then there must be an interesting mechanism for maintaining a static position for so long. Second, the deliveries of endocytosed modeccin to lysosomes and to the cytosol were both apparently blocked at low temperature. Although this correlation could be a coincidence, it suggests that modeccin traveling to the cytosol may enter a path, blocked at low temperature, that carries material from endosomes to lysosomes. This possibility is consistent with the approximate 30-min interval before modeccin reached the cold-sensitive step since 30 min is within the time usually needed for extracellular ligands to reach lysosomes (19). Thus, part of the 75 min required for modeccin to enter the cytosol could be accounted for by a trip through lysosomes. Other possibilities, however, are not excluded by our data; for example, to reach the cytosol, modeccin may have to travel from endosomes to some other intracellular compartment (Golgi?) by a route that could also be blocked at low temperature.

Exactly what happens to modeccin as a result of the acidification step is unknown, but several properties of this step can be inferred from the available data. Since modeccin passed the acidification step at 15 °C, this event can presumably occur within a prelysosomal vesicle. After modeccin had passed the acidification step at 15 °C, an elevation of intravesicular pH caused by ammonium chloride no longer blocked the activity of the toxin at 37 °C (Table I). This observation suggests that the acidification step is an irreversible event and that once this step has happened, subsequent steps are independent of the pH inside vesicles, whether the toxin passes through lysosomes or not. Since Sandvig and Olsnes (8) showed that the acidification step does not occur when modeccin on the cell surface is exposed to acidified medium, the toxin must apparently reside within a vesicle to pass the acidification step. There is an interesting difference between modeccin and diphtheria toxin regarding the kinetics of acidification; diphtheria toxin is acidified within a minimum of 4 min after being endocytosed by Vero cells, while 15 min elapsed between the endocytosis and acidification steps for modeccin. It may be that modeccin first enters the cell in vesicles whose interior is at neutral pH and that 15 min is needed to reach acidic vacuoles. Alternatively, modeccin may quickly encounter a low pH but not respond for some reason until 15 min later.

If modeccin does pass into lysosomes before the A chain enters the cytosol, something must protect it from degradation. One possibility is that toxin molecules en route to the cytosol have only transient residence within lysosomes and migrate to another compartment before they can be destroyed. That some membrane-bound ligands could survive a trip through lysosomes is indicated by the work of Schneider et al. (20) who found that antibodies to plasma membrane antigens entered lysosomes and were returned to the cell surface. Muller et al. (21) also noted that membrane proteins initially within secondary lysosomes of macrophages were rapidly redistributed to the plasma membrane. It could be that modification of modeccin during transient exposure to lysosomal hydrolases is even necessary to generate a penetration-competent form of the toxin. This would explain why modeccin fails to penetrate the membrane of a prelysosomal vacuole after passing the acidification step at 15 °C since the toxin would not have been exposed to lysosomal enzymes. If lysosomal enzymes do generate a penetration-competent form of modeccin, they are able to do so even though the pH within lysosomes has been elevated by ammonium chloride because the addition of ammonium chloride at 31 °C to cells that had been previously incubated with the toxin at 15 °C did not block cytotoxicity. The possibility that lysosomal enzymes could retain activity even in the presence of ammonium chloride is not unreasonable; for example, diphtheria toxin is degraded and excreted by Vero cells with similar kinetics in the presence and absence of ammonium chloride (22).

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REFERENCES

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