Biochimica et Biophysica Acta, 323 (1973) 87–97

BBA 76437

A COMMON RECEPTOR PROTEIN FOR PHAGE T5 AND COLICIN M IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI* B

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(Received June 18th, 1973)

SUMMARY

The receptor protein for phage T5 was isolated from the outer membrane of *Escherichia coli* B and found to be also a receptor for colicin M. The receptor protein from a phage-resistant mutant inactivates neither the phage nor the colicin. Binding of colicin M to the receptor prevents binding of phage T5. It is concluded that phage T5 and colicin M bind to the same active area of this receptor protein. The receptor protein seems to consist of one polypeptide chain with a molecular weight of 85000.

INTRODUCTION

Bacterial viruses as well as bactericidal proteins, *e.g.* colicins, can be regarded as being membrane effectors acting first on the bacterial cell surface. For example DNA-free phage T4 ghosts kill sensitive bacteria[1] as do colicins[2]. The killing of cells requires specific receptors on the cell surface. Depending on the effector used, a large variety of different subsequent events have been observed such as inhibition of protein, RNA or DNA synthesis[1,3], degradation of DNA and RNA[4–7], inhibition of active transport[1,3,19], loss of intracellular potassium or ATP[8–10], *etc.* It has been proposed that colicins remain bound to the receptor and act from there[11,12]. This comes from the observation that at least 90% of colicin E2 can be found in the membrane after adsorption and that inhibition of macromolecular synthesis by colicin K can be reversed by trypsin which does not penetrate into the cell. This implies that binding to the outer membrane somehow triggers events in the inner membrane or the cytoplasm which lead to cell death. In fact, when colicin E1 binds to sensitive cells it causes a blue shift and a 100% increase in the fluorescence-emission spectrum of the cell-bound fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS), probably reflecting conformational changes in the cell envelope[9,10]. Envelope fractions which strongly bind colicins E2, E3[13], and K[13,14] have been isolated and the receptor for colicin E3 has been purified more extensively[15]. However, recent studies show that colicin E3 mediates directly the specific cleavage of about 50 nucleotides from the 3'-end of the 16-S rRNA when mixed with isolated ribosomes and thus inhibits protein synthesis[5–7]. From this one has to conclude that colicin E3 has its target in the cytoplasm despite the fact that it must first bind to the membrane.

To obtain more insight into the membrane aspects of the interactions between

colicins and phages and sensitive cells, study of isolated receptors is needed. The receptor of the phage T5 seemed to be particularly suitable since when isolated in crude form[16] it contained protein, lipid and lipopolysaccharide. Therefore, it is feasible that the arrangement of these major components of the outer membrane and their mode of interaction could be studied with purified T5 receptor with the aim of elucidating the function of a whole membrane area. In addition, genetic studies[17,18] showed that phage T5 and colicin M share a common receptor. We first attempted to isolate the active component of the "receptor area". In the following it will be shown that this is a protein and that both phage T5 and colicin M bind to the same polypeptide chain.

MATERIALS AND METHODS

Strains and culture conditions

Escherichia coli B and phage T5 are from the collection of the late W. Weidel[20]. Phage T5 was grown on *E. coli* B in M3 medium (Difco) supplemented with 10^{-3} M $MgCl_2$ and 10^{-3} M $CaCl_2$ and was purified by precipitation with polyethyleneglycol[21].

The colicin M-producing *E. coli* K12 32 T 19/ V, T5, is Met^-, Str^R, $ColV^R$, $T1^R$, $T5^R$, is λ-lysogenic and carries the Col M plasmid from *E. coli* K260. Later a non-lysogenic derivate, *E. Coli* 32 T 19 F/T1, was obtained from P. Fredericq. *E. coli* K12 ROW/V/22.1 is Met^-, Str^R, $ColV^R$, $ColB^R$, λ-lysogenic and served as indicator strain to test colicin M. These strains were isolated by P. Fredericq, Liège, and two of them were obtained through B. Stocker, Stanford. The colicin M-producing strain was grown in M9 salt medium (42 mM $Na_2HPO_4 \cdot 2\ H_2O$, 22 mM KH_2PO_4, 8.5 mM NaCl, 1.87 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.4% glucose, 1% yeast extract (Merck, Darmstadt) and 20 mg/l L-methionine to late logarithmic growth phase. Isolation and purification of colicin M will be described in a subsequent paper. The indicator strain was grown on nutrient agar plates (Difco) supplemented with 1% tryptone, 0.5% yeast extract (Difco), 0.5% NaCl. The same plates were used for determining the phage titer on *E. coli* B.

Spontaneous *E. coli* B mutants, resistant to phage T5, were isolated by seeding $3 \cdot 10^8$ bacteria and 10^9 phages in 2.5 ml molten nutrient soft agar onto nutrient plates. After 1 day of incubation at 37 °C about 10 well-grown colonies were obtained which were grown in liquid nutrient broth and streaked again on minimal agar plates.

Isolation of the T5, colicin M receptor

50 g (wet wt) of *E. coli* B, freshly grown aerobically to the late logarithmic growth phase in M9 salt medium with 0.2% glucose at 37 °C, were suspended in 250 ml water. 125 ml 0.1 M NaOH were added dropwise at 25 °C and then CO_2 gas was passed through until the pH reached 6–7 (ref. 20). After incubation with 3 mg of deoxyribonuclease for 10 min, the suspension was centrifuged for 1 h at $16000 \times g$ at 4 °C. The pellet was extracted again with NaOH as above and the combined supernatant solutions concentrated with the Amicon ultrafiltration system to 200 ml. The receptor was spun down at $80000 \times g$ in the SW27 rotor for 15 h at 4 °C, suspended again by ultrasonic treatment in 5 ml 0.01 M sodium phosphate buffer,

pH 7.0, (containing 0.1% sodium azide to prevent microbial growth). Remaining cells and large particles werd spun down at 3000 × *g* (10 min).

Chromatography of receptor

Biogel A50: The receptor solution (5 ml) obtained from 50 g of cells was chromatographed on a 90 cm × 3 cm Biogel A50 (BioRad, München) column with 0.01 M sodium phosphate, pH 7.0, at 4 °C. 4 fractions/h with a volume of 5 ml were collected. 0.3 ml of every second fraction was hydrolysed with 1 ml 2.5 M NaOH and stained with ninhydrin. For determination of receptor activity 0.1-ml aliquots of the fractions were mixed with 0.8-ml adsorption buffer (0.01 M sodium phosphate, pH 7, 0.01 M $MgSO_4$) and with 0.1 ml of a suspension of about 10^3 T5 phages and incubated for 1 h at 37 °C. 0.2 ml of the incubation mixture was mixed with 0.2 ml of stationary phase *E. coli* B in 2.5 ml of soft agar and plated on nutrient agar (1% tryptone, 0.5% yeast extract, 1% agar from Difco, 0.5% NaCl). The number of plaques were compared with those of the control plates where the same number of phages were mixed with 0.1 ml of elution buffer instead of fraction aliquots. The peak fractions containing the soluble receptor will be henceforth called "T5 receptor".

DEAE-cellulose

The fractions containing soluble receptor (No. 90–115, Fig. 1) from three Biogel A50 runs were combined, concentrated by ultrafiltration and the material, which partially precipitated, dispersed again by adding Triton X-100 to a final concentration of 2%. After ultrasonic treatment, the solution was briefly centrifuged and the supernatant (25 ml) applied to a DEAE-cellulose column (56 cm × 3 cm), equilibrated with 0.01 M sodium phosphate, pH 7 which contained 2% Triton X-100. Equilibration buffer containing increasing amounts of NaCl (0.1 to 0.5 M) was pumped into a 250-ml mixing vessel which initially contained the equilibration buffer and from there it was placed onto the column. Protein and receptor activity were determined as described for the Biogel A50 run. Receptor activity was found in Fractions 75–210. Fractions 30–62, 75–80, 88–99, 100–112, 116–131, 133–170, 180–230 were collected separately, concentrated by ultrafiltration under nitrogen pressure and then lyophilized. They were then taken up in 5 ml water and freed from Triton X-100 by precipitating the protein with 30 ml ethanol at 0 °C. The precipitates were washed twice with ethanol and then lyophilized.

Gel electrophoreses

(1) The number of proteins in a given receptor preparation and their molecular weight was estimated by gel electrophoresis. 0.35 mg of salt-free lyophilized receptor was dissolved with the aid of brief ultrasonic treatment in 0.2 ml sample buffer (6 M urea, 1% sodium dodecyl sulfate, 1% mercaptoethanol, 0.01 M EDTA, 0.01 M sodium borate, pH 8). The gels and the electrode chambers contained 0.01 M sodium borate, 0.005 M EDTA, 0.1% sodium dodecyl sulfate, pH 8. This buffer system was developed by H. Müller of this laboratory who also ran the gels of Fig. 4. The gel was prepared with 6% acrylamide, 0.12% methylenebisacrylamide, 0.14% *N,N,N′,N′*-tetramethylenediamine, and 0.045% ammonium persulfate and contained in addition to the buffer, 6 M urea. Electrophoresis was performed

at a constant current of 3 mA/gel. Otherwise the procedure followed that of Weber and Osborn[26].

(2) For reisolation of receptor bands from sodium dodecyl sulfate gels the sample buffer contained 0.1 M Tris, 0.1 M EDTA, 1% sodium dodecyl sulfate, pH 7.9, which was diluted ten times for use as gel and electrode buffer. 20 gels were usually run simultaneously from which 2 were stained to localize the protein bands. The protein bands were cut out of the unstained gels, eluted overnight with water and the extract either used for determination of activity or subjected to amino acid analysis after hydrolysis with 4 M HCl for 15 h at 105 °C.

(3) In early experiments for localizing receptor activity in the protein bands after gel electrophoresis, Triton X-100 was used instead of sodium dodecyl sulfate. The electrophoresis procedure was otherwise than that described in (1) but with the omission of urea. The recovery of the receptor protein was performed as indicated in (2).

Amino acid and amino sugar analyses

They were performed as previously described[23]. The presence of lipid was estimated by thin-layer and gas chromatography as published previously[24]. The determination of lipopolysaccharide was based on the amount of ketodeoxyoctonate and β-hydroxymyristic acid[25] and on the amount of glucosamine, the latter determined with the amino acid analyser.

RESULTS

Isolation of the receptor

Receptor activity can be measured by inactivation of phage. Phage T5 irreversibly attaches to the receptor and releases DNA[16,27]. The receptor was extracted from freshly grown *E. coli* B with NaOH as previously described by Weidel[20]

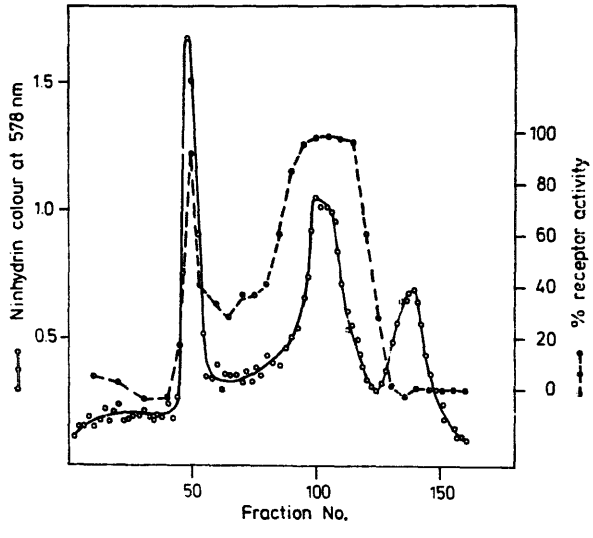

Fig. 1. Chromatography of receptor on Biogel A50. The receptor-containing extract from 50 g of cells was dissolved after differential centrifugation in 5 ml 0.01 M sodium phosphate, pH 7, and chromatographed on a 90 cm × 3 cm column of Biogel A50. The protein concentration was determined with the ninhydrin reaction after alkali hydrolysis of fraction aliquots. Receptor activity was determined as the reduction of the number of viable phages (plaque-forming units) when incubated with fraction aliquots. Total inactivation of 10^8 phages by 0.1 ml of the fractions was arbitrarily taken as 100% receptor activity.

and further fractionated by differential centrifugation and gel chromatography on Biogel A50 (Fig. 1). Apart from the NaOH extraction all the other steps of the former preparation procedure were avoided for reasons discussed later. The first peak with receptor activity contained membrane fragments and was, therefore, not studied further. The second peak was a clear solution. When the collected fractions were subjected to DEAE-cellulose chromatography with a linear salt gradient at pH 7, the receptor activity together with all the protein was eluted as a single peak at 0.3 M NaCl. However, with 2% Triton X-100 the receptor particle could be dissociated into smaller fragments which were separable on DEAE-cellulose (Fig. 2). All protein peaks except the first contained receptor activity.

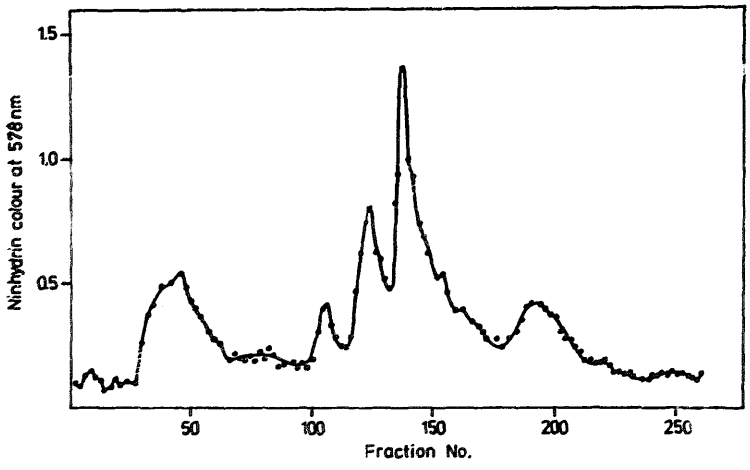

Fig. 2. Chromatography of receptor on DEAE-cellulose. Fractions 90–115 of Fig. 1, containing the soluble receptor, were chromatographed on DEAE-cellulose with the solvent 2% Triton X-100, 0.01 M sodium phosphate, pH 7.0, and increasing concentrations of NaCl (logarithmic gradient). Fractions 75–230 contained active receptor. The curve given expresses the protein concentration in the fractions.

As seen by sodium dodecylsulfate gel electrophoresis (Fig. 3), all active peaks had one protein band in common. In one peak (Fractions 75–80) only this protein band was present. To prove that this protein band inactivates T5 phages, gel electrophoresis was performed in 2% Triton X-100, the band cut out, eluted with water and tested with phage T5. Based on the protein content the recovered substance inactivated as many phages as prior to electrophoresis.

Characterization of the receptor

The soluble receptor after chromatography on Biogel A50 contained protein, phospholipid and lipopolysaccharide. The same components were still present in the peak fractions of the DEAE chromatography (Fig. 2) and in the active protein band after gel electrophoresis in the presence of Triton X-100. In the latter case, however, phospholipids were greatly reduced. The question arose whether the activity resides in the protein or the lipopolysaccharide (phospholipid) or whether all components were necessary. In the crude receptor fraction, isolated earlier[16], phenol destroyed T5 receptor activity but uncovered receptor activity for the phages T3, T4 and T7[36]. It was concluded that the latter phages bind to lipopolysaccharide in agreement with the results in recent papers (*e.g.* see ref. 28). In the case of phage T5 the conclusion was drawn that phenol denatures a protein essential for T5

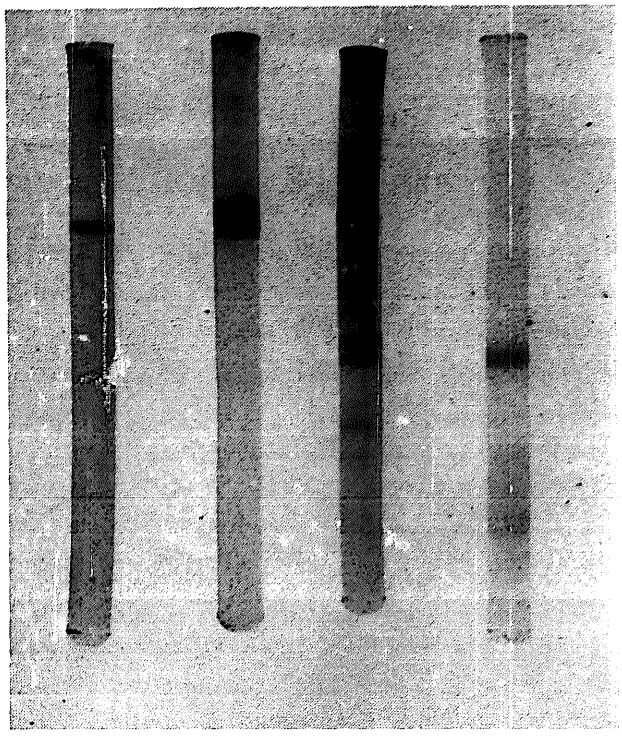

Fig. 3. Polyacrylamide gel electrophoresis of receptor fractions. Gels from left to right: From the DEAE-cellulose chromatogram (Fig. 2) the bulked fractions, 75–80, 100–112, 116–131, 30–62. Fractions 133–170 and 180–230 show similar protein band patterns as Fraction 116–131 and are probably complexes of the same composition but with more units of each constituent. The Electrophoresis System I (see Materials and Methods) was used.

receptor activity. However, the earlier isolation procedure of active receptor involved a lengthy treatment with a mixture of hydrolases including proteases (Pancreatin, Merck). That proteases had been active during this incubation can be seen from the fact that the nitrogen content of the receptor particle fraction was reduced from 25 μg/unit receptor to 0.7 μg/unit receptor[20]. The argument, based on phenol sensitivity, that a protein is essential for the receptor activity was, therefore, questionable.

To clarify this contradiction, we digested our highly purified receptor, showing only one protein band in Triton- and sodium dodecylsulfate gel electrophoresis (Fig. 3, left gel) with various proteases. The proteases were selected for easy inactivation before phage T5 was added to the receptor. The capacity of the receptor to inactivate T5 was reduced to 52% with trypsin and was completely destroyed with subtilisin (Table I). This shows that a protein is essential. By oxidation with sodium periodate we tested whether periodate-sensitive sugars bound to protein or lipid are involved in phage inactivation. As seen from Table II, receptor activity was fully retained.

The protein was still contaminated with lipopolysaccharide and attempts to extract lipopolysaccharide from a 2% Triton X-100 solution with ethanol failed. The receptor remained active and lipopolysaccharide was not removed from the protein. Extraction with acetone or phenol destroyed the activity irreversibly. However, separation was successfully achieved by sodium dodecyl sulfate gel electrophoresis. As shown in Table I, 0.5% sodium dodecyl sulfate does not destroy

TABLE I

INACTIVATION OF RECEPTOR BY PROTEASES AND CHEMICALS

200 μg of Receptor Fraction 100–112 (Fig. 2) in 0.5 ml 0.012 M Tris –HCl, pH 8.0, were incubated for 3 h at 37 °C with 0.1 mg trypsin or subtilisin. The reaction was stopped by addition of 0.1 mg phenylmethylsulfonylfluoride in 0.1 ml ethanol, or also, in the case of trypsin, by adding 1 mg soy-bean trypsin inhibitor or by adding Triton X-100 to a final concentration of 2%. After further incubation for 30 min at 37 °C, 0.25 ml were mixed with about 10^3 T5 phage in 0.75 ml 0.01 M sodium phosphate buffer, pH 7.0, 0.01 M $MgSO_4$ (adsorption buffer) and incubated for 60 min at 37 °C. The protease inhibitors do not affect phage viability. Receptor was incubated with the chemicals at concentrations indicated for 30 min at 25 °C, diluted four-fold before phage T5 was added. Since phage T5 is destroyed by sodium dodecyl sulfate concentrations greater than 0.025%, the sodium dodecyl sulfate solution was dialysed against the adsorption buffer before the receptor activity was measured.

Receptor treated with	*Receptor activity*
—	100%
Trypsin	52%
Subtilisin	0%
2% Triton X-100	100%
0.5% sodium dodecyl sulfate	100%
0.1 M urea	65%
0.5 M urea	48%

TABLE II

EFFECT OF PERIODATE ON THE T5 RECEPTOR ACTIVITY

A_1, A_2: 2 samples of T5 receptor were incubated at pH 4.5 and pH 7 with sodium metaperiodate[22] for 1.5 h, excess periodate was destroyed with glucose and then the samples were dialysed overnight B–D controls: B, periodate and glucose were first incubated for 1 h before receptor was added; C, receptor solution without periodate and glucose added; D, periodate and glucose as in B, without receptor added to test for phage inactivation without receptor. Receptor activity was tested with about 100 T5 phages.

Number of plaques (2 plates from each sample)				
A_1	A_2	B	C	D
2; 3	1; 3	1; 1	2; 1	96; 110

receptor activity and after dilution to 0.025% sodium dodecyl sulfate phage survival is also unaffected. Therefore, the receptor was run on twenty sodium dodecyl sulfate gels, the protein band was eluted with water and half of it hydrolyzed to test for glucosamine and to determine the amount of protein with the amino acid analyser. The receptor activity was tested with the other half. Since no glucosamine was found, lipopolysaccharide was absent. Based on the protein content, the lipopolysaccharide-free protein band inactivated as many T5 phages as prior to electrophoresis when lipopolysaccharide was present. We, therefore, conclude that the receptor activity resides solely in this protein band, that the protein is accessible to the phage in the presence of the lipopolysaccharide and that the latter is also not

necessary to stabilize the protein conformation. The fact that the protein is active in sodium dodecyl sulfate concentrations where it should be fully covered with the detergent[33] is surprising and needs further studies. Also 2% Triton X-100 apparently does not interfere with phage–receptor binding. Instead it enhances receptor activity by dissociating the receptor which has a tendency to aggregate. Up to 1 M urea does not inactivate the phage but urea does interfere with receptor–T5 interaction at a concentration of 0.025 M. From these preliminary observations we conclude that polar or hydrogen bonds rather than hydrophobic bonds are primarily involved in the specific binding of T5 to the receptor protein.

Sodium dodecyl sulfate gel electrophoresis of receptor protein with standard proteins of known size revealed a molecular weight of about 85000 which was not changed by heating prior to electrophoresis. The electrophoretic mobility of some *E. coli* membrane proteins is increased by heating[29–31]. Nevertheless, this molecular weight estimation has to be taken with caution since it is possible that the receptor protein is a glycoprotein and glycoproteins can move slower in sodium dodecyl sulfate gels than pure proteins of the same real molecular weight[32]. The receptor activity has been localized in the outer membrane after separating the cytoplasmic and outer membrane according to the procedure of Osborn *et al.*[34] (data not shown).

Interactions of phage T5 and colicin M with receptor

Mutants have been isolated which are resistant to phage T5 and colicin M simultaneously[17] but later these two events were not considered to be strictly correlated[35]. In a more recent paper[18], genetic evidence was again presented which supported the common receptor hypothesis. The basic problem seems to be the unequivocal proof that the producer strain makes only one colicin and that the indicator strain is only sensitive to this colicin. This is especially true for ill-defined colicins where no biochemical investigations have been performed as is the case with colicin M.

To be sure that we work with one colicin, we purified the colicin from a cell homogenate to homogeneity. Purity was tested in various gel electrophoresis systems with and without sodium dodecyl sulfate. Only one protein band was observed (Fig. 4, right gel). To test whether colicin M binds to the isolated T5 receptor, we determined the residual colicin activity after incubation with the isolated T5 receptor. The colicin activity was determined with the spot assay[41] with the colicin concentration barely high enough for a clear spot to appear on a confluent layer of the indicator strain. As little as 30 μg/ml of T5 receptor inactivated enough colicin M, so that a turbid spot was obtained instead of a clear spot. 300 μg/ml abolished spot formation completely. To test for the specificity of the reaction, we isolated a spontaneously resistant mutant of *E. coli* B to which phage T5 did not adsorb, and prepared the protein fraction corresponding to the soluble active receptor fraction from sensitive cells (Fig. 1). The strict reproducibility of the protein profile of the Biogel A50 column chromatography allowed this blind isolation. The protein band corresponding to the receptor protein from sensitive cells was present with the same molecular weight but no reduction of viable T5 phages (plaque-forming units) was observed even when ten times the normal amount of protein was used. Similarly, when the inactive receptor was incubated with colicin M, no reduction of colicin M activity was observed. When colicin M was preincubated

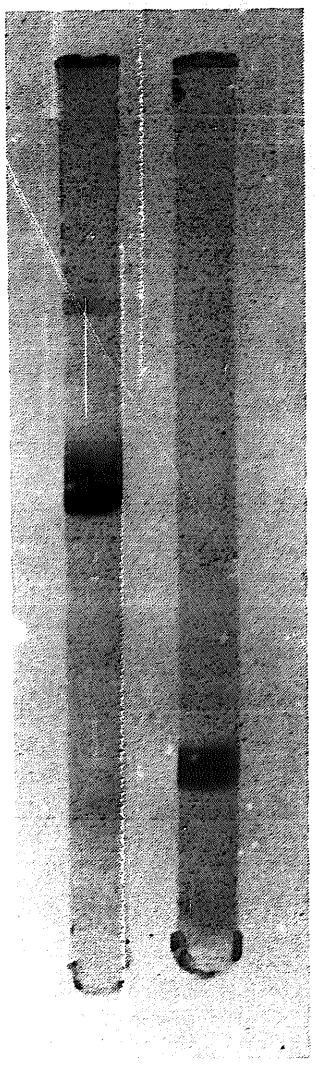

Fig. 4. Comparison of the protein pattern of the phage T5 with colicin M. $6 \cdot 10^9$ T5 phages dissolved by boiling 5 min in Buffer System II (see Materials and Methods) were applied to the left gel which consisted of a gel with 10% acrylamide and Buffer II. Colicin M was dissolved in Buffer II without heating and run on the right gel. The faint fast moving band in the T5 protein pattern is due to added cytochrome *c*.

with active T5 receptor and then phage T5 was added in the salt medium used for adsorption, only about 10% of the phages were inactivated by the receptor compared to the control without colicin M. Colicin M thus seems to bind to the same receptor area as the phage. This is strongly supported by the fact that in the inactive receptor the protein is still there with the same molecular weight pointing to a minute change as an amino acid exchange. Studies of the primary structure will be necessary to reveal the difference between the active and the inactive receptor protein.

The interesting question arises whether colicin M has something in common with or even arose from a tail-tip protein of phage T5. Preliminary gel electrophoreses of the phage proteins dissolved by heating in sodium dedecyl sulfate show no protein band with a molecular weight like colicin M (Fig. 4, left gel). The small band in the phage pattern running somewhat faster than colicin M (Fig. 4, right band) is added cytochrome *c*. A high titer antiserum, from which 0.1 ml inactivates more

than 10^8 phage particles, does not inactivate colicin M. These observations argue against the hypothesis that colicin M may be derived from a protein at the tail tip of the phage responsible for adsorption of the phage to sensitive cells.

DISCUSSION

This paper provides biochemical evidence for the existence of a common receptor for a phage and a colicin. The receptor activity is manifested by an isolated single polypeptide chain. The most convincing evidence for a common receptor is that in a spontaneous mutant, simultaneously resistant to the phage and the colicin, the protein is still present but inactive. Other reasons for simultaneous resistance conceivable with whole cells, such as steric hindrance of a whole membrane area by structural changes not related to the actual receptor, are thus in this case excluded. Since the inactive receptor protein apparently has the same molecular weight as the active one (small deletions would not have been detected), it is likely that an amino acid difference or some similar small change is responsible for inactivation. This would mean that phage T5 and colicin M not only bind to the same polypeptide chain but that they also bind to the same "active center". Since our studies so far do not convincingly exclude the possibiliy that the receptor protein is a glycoprotein, the difference between the active and inactive form could also reside in the sugar moiety.

Until now, no evidence has been found that colicin M may be related to a tail-tip protein of phage T5. But the evidence is not conclusive. The antibody population tested for activity against colicin M may be directed against the most abundant phage head protein(s) and the major tail protein(s) and not against a tail-tip protein from which only a few copies may exist. A few copies of a tail-tip protein would probably also have escaped recognition in the gel electrophoresis of the complete phage. Antisera against purified colicin M, however, should block phage adsorption to cells provided structural similarities exist between the colicin and the tail-tip protein. Studies to test this are under way.

When phage T5 binds to sensitive cells, the release of DNA is triggered. This process can be imitated with the isolated receptor[27]. Transfer of DNA into the cytoplasm is in the case of phage T5 a two-step process[37,38]. We can now study triggering of DNA release with the isolated receptor protein, the solubilized receptor area of the membrane, the complete cell envelope, and the cell, with the aim of obtaining information about the molecular processes involved.

It seems clear that living cells possess specific receptors for binding colicins[2,11–15,18]. It is likely that receptor-bound or additionally adsorbing colicin molecules move within the membrane to the real target which may be localised within the membrane or in the cytoplasm. Structural transitions in the membrane caused by the binding to receptor may be the prerequisite for colicins to reach their target. A multi-stage process of colicin action is suggested from several types of experiments[10,39,40].

ACKNOWLEDGEMENT

We thank Drs P. Fredericq and B. Stocker for very helpful information and for providing us with bacterial strains. We are grateful to Mark Achtman for reading the manuscript.

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