PHAGE λ INTEGRATION PROTEIN: SYNTHESIS IN λ-INFECTED MINICELLS AND MEMBRANE AFFINITY

Elżbieta GRZESIUK[+,*], Maciej ŻYLICZ, Barbara LIPINSKA and Karol TAYLOR
Departments of [+]Microbiology and Biochemistry, University of Gdańsk, 24 Kladki, 80-822 Gdańsk, Poland

Received 8 April 1980
Revised version received 8 May 1980

1. Introduction

The integration of phage λ DNA into the chromosome of *Escherichia coli* [1,2] requires participation of host and phage proteins. The nature of bacterial proteins has not been clarified yet; they seem to correspond to at least two genetic loci known to be important for λ integration [3]. The *int*-gene product appears to be the only phage protein that plays a direct role in the integration. Int protein was identified by SDS gel electrophoresis of proteins synthesized after infection of bacteria whose endogeneous protein synthesis had been inhibited by prior ultraviolet irradiation [4–7]. Int isolated from UV-treated cells does not form stable complexes with double-stranded λ DNA containing an attachment site, the region at which recombination takes place [5,6]. When UV-irradiation was omitted, Int with a complex-forming ability [8], active in the in vitro integrative recombination [9,10] could be isolated. Presumably, the Int synthesized in UV-treated cells was damaged, or became damaged during purification [1]. It seems, therefore, that the lysates of irradiated bacteria do not represent a suitable material for studying the physical interactions of Int with other recombination components.

An alternative approach to the identification of λ-coded proteins is the study of λ-infected anucleate minicells of *E. coli* [11,12]; moreover, in this method the results are more amenable to interpretation, due to the extremely low background of host protein synthesis. Here we present evidence that the synthesis of Int protein in minicells occurred only when λ mutant expressing *int* constitutively was used for infection. By applying two different methods of minicell lysis and lysate fractionation, which have in common an avoidance of detergents, Int was found exclusively in the membrane fraction. The binding of Int to the membrane was salt-resistant and detergent-sensitive.

* Present address: Polish Academy of Sciences, Institute of Biochemistry and Biophysics, 36 Rakowiecka, 02-532 Warsaw, Poland

2. Methods

The phages λ*cIts857Sam7* and λ*cIts857intc226Sam7* [13] (called λ and λ*int*c in the text) were purified by banding in CsCl gradient.

Minicells were isolated, purified [14] and infected with phage λ, as in [12], at a multiplicity of infection (m.o.i.) of 10. Proteins were ^{14}C-labelled with radioactive amino acids and separated by 10% polyacrylamide–SDS slab gel electrophoresis, as in [12], except that labelling was performed at 37°C and that 15 cm long slabs were used. Radioactive proteins synthesized in minicells were detected by fluorography [15] and traced with a Vernon densitometer. The identification of proteins coded by the genes *E*, *V* and *P* was performed by the infection of nonpermissive minicells with the appropriate amber mutants of λ [16,17].

At first minicell lysis and lysate fractionation were performed according to [18]. The minicells were suspended in 50 mM Tris–HCl buffer (pH 8.0), 2 mM EDTA, containing 300 μg/ml of phenylmethylsulfonyl fluoride (PMSF) and disrupted by incubation with egg lysozyme (40 μg/ml, 20 min, 4°C) and sonification. The lysate was incubated with pancreatic DNase and RNase for 30 min at 4°C, and submitted to centrifugation (Beckman 50Ti rotor with 2.5 ml adapters) in order to separate membrane-associated proteins from the soluble ones.

Later we elaborated another method of minicell

lysis and lysate fractionation [19]. After ^{14}C-labelling with radioactive amino acids, the phage-infected minicells were suspended in the cold (0°C) lysis buffer (50 mM Tris–HCl (pH 7.3), 40 μM spermine, 1 mM EDTA, 5 mM dithiothreitol, 10 μg/ml *E. coli* tRNA, 100 μg/ml PMSF), additionally containing saccharose (10%, w/v), and were frozen at −70°C. After thawing in an ice-water bath and addition of T4 lysozyme to 2 μg/ml final conc., the suspension was incubated for 30 min at 0°C, then diluted 2-fold with the cold lysis buffer. The unlysed minicells (<10% of the initial amount) were removed by sedimentation (60 s, 0°C) in the Eppendorf centrifuge. The lysate was made 50% (w/v) in metrizamide and fractionated by sedimentation to equilibrium in a preformed 25–50% (w/v) metrizamide gradient (Beckman 50Ti rotor, 20 h, 5°C, 40 000 rev./min). The free proteins remained in the lower part of the tube (d = 1.24–1.27 g/cm^3) and the membranes moved nearly to the middle of the gradient (d = 1.18–1.22 g/cm^3) [19].

3. Results and discussion

No Int synthesis could be detected in λ-infected minicells (fig.1A), even by using high m.o.i. Most probably, the activation of *int*-gene expression by the *c*II and *c*III gene products [20] is ineffective in minicells. Since this activation seems to be replication-dependent [21,22], one of the reasons of such inefficiency could be the absence of λ DNA replication in minicells [23]. An additional regulatory block may exist in minicells, since in UV-treated bacteria a slight expression of *int*, presumably from the promoter p_L, takes place even in the absence of *c*II/*c*III activation [7,22].

We could observe Int synthesis in minicells only when λ mutant expressing *int* constitutively was used for infection (fig.1B). This finding makes it possible to ask detailed questions about how the Int protein interacts with DNA and with host structural elements present in minicell lysates. Here we provide evidence that Int has affinity to the minicell membrane.

At first we adopted the method used in [18] for the disruption of minicells and for the separation of the membrane-associated proteins from the soluble proteins, after incubation with nucleases. The minicell lysis was complete, as judged from experiments including initial short centrifugation (60 s, 0°C, Eppendorf centrifuge) of the lysate. One of the λ-coded

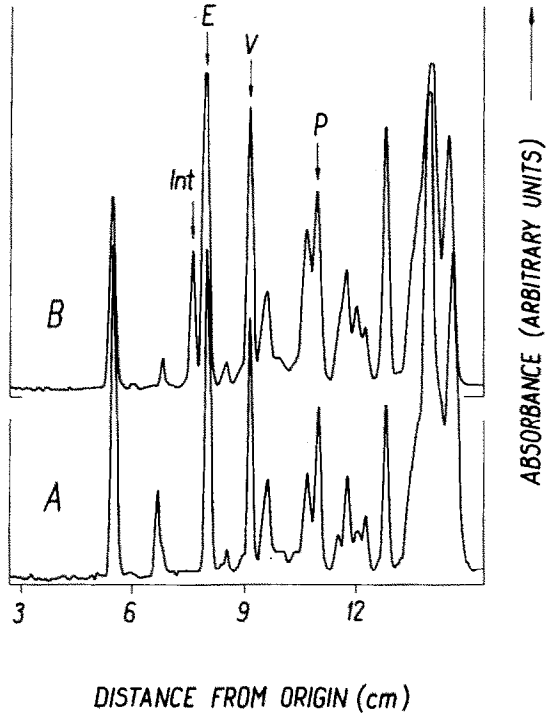

Fig.1. SDS–acrylamide electrophoresis of proteins synthesized in minicells infected with phage λ (A) or λ*int*c, a mutant expressing *int* constitutively (B). λ-sensitive and maltose-fermenting derivative A127, selected from the minicell-producing strain P678-54 [ColE1] was used as a source of minicells; they were purified as in [14]. The phages were purified by banding in CsCl gradients. To the suspension of minicells (10^{10}/ml) in the adsorption buffer, the phage was added at m.o.i. 10. After 5 min adsorption at 0°C, 10 vol. preheated (37°C) TMM medium containing [U-^{14}C]aminoacid mixture (57 mCi/matom, Amersham CFB.104) at 10 μCi/ml were added. The TMM medium was 0.1 M Tris–HCl buffer (pH 7.2) containing 10 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% NH_4Cl, 0.05% NaCl, 1% maltose, 4 μg/ml L-threonine, and 2 μg/ml of: L-leucine, L-tryptophane, L-cysteine, L-histidine, L-methionine and thiamine hydrochloride. After 30 min incubation at 37°C, the suspension was chilled and the λ-infected minicells were harvested by centrifugation, washed with cold TMM medium and stored frozen at −20°C. The radioactive proteins were separated by 10% acrylamide–SDS electrophoresis [12] in 15 cm long slab gels, detected by fluorography [15] and traced densitometrically. The proteins coded by the λ genes *E*, *V* and *P* (38 000, 31 000 and 23 000 daltons, respectively) were identified by parallel runs of the proteins synthesized in the λ*Eam*-, λ*Vam*- or λ*Pam*-infected minicells nonpermissive for amber mutants [16,17]. The molecular weight of the additional protein coded by the *int*-constitutive mutant (~40 000 daltons) agrees with the estimated molecular weight of Int protein [4–7].

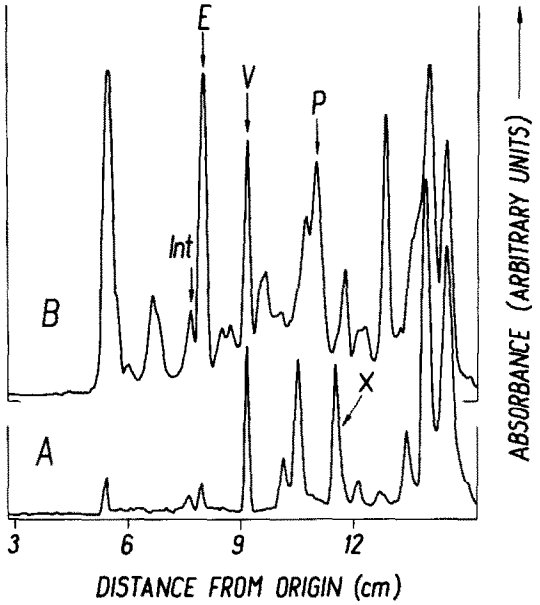

Fig.2. Electrophoretic pattern of proteins synthesized in λ*int*c-infected minicells and present in the supernatant (A, soluble proteins) and in the sediment (B, membrane-associated proteins) of the minicell lysate obtained according to [18]. ^{14}C-Labelling of proteins synthesized in phage-infected minicells, their electrophoretic separation, detection by fluorography and identification was performed as in the legend to fig.1. The designation of protein peaks is the same as for fig.1; X represents a λ-coded protein present exclusively in the supernatant.

proteins, designated X in fig.2, was present exclusively in the supernatant (soluble proteins), proving that the sediment (membranes) was not contaminated by unlysed minicells. Some proteins (e.g., the major protein of the phage tail, coded by gene *V*) were present in both fractions. However, the Int protein, as well as the λ DNA replication protein coded by the gene *P* [17] was found exclusively in the sediment (fig.2). A comparison of two neighbouring peaks, representing Int and the gene *E* product (major phage head protein) in fig.1,2, suggests that Int underwent considerable degradation during the experiment, in spite of a high concentration of PMSF, an inhibitor of serine proteases. Independent experiments (results not shown) have demonstrated that this degradation occurs during incubation of the minicell lysate with nucleases.

Only small amounts of Int and of the *P*-gene product could be eluted by 0.125 M KCl. The application of successive higher concentrations of KCl (0.25 M, 0.5 M, 1.0 M and 2.0 M) were without effect: even when washing with 2.0 M KCl, large amounts of these two proteins were present in the sediment (not shown). It is worth mentioning that most of the Int protein was recovered in the 'cleared lysate' prepared from λ-infected minicells by the method in [24] (not shown). Since this method of lysis consists in the action of EDTA-lysozyme followed by incubation with 0.5% Brij-58 and 0.2% deoxycholate, it is obvious that the detergents used caused elution of this protein from the membrane structures.

We have developed a more gentle method of opening the minicells without detergents; this method consists in the addition of T4 lysozyme at a low concentration. The minicell lysate is subsequently fractionated by equilibrium sedimentation in a metrizamide density gradient at a low ionic strength [19]. The phage λ proteins, synthesized in minicells, form two main peaks in this gradient, corresponding to free proteins and to the membrane-bound proteins. When the

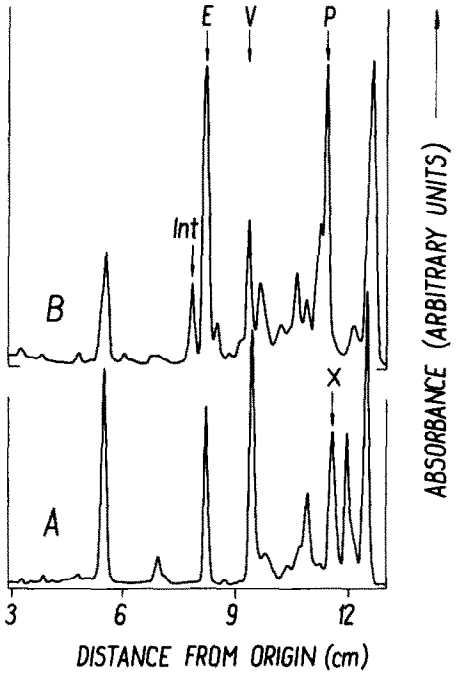

Fig.3. Electrophoretic pattern of proteins synthesized in λ*int*c-infected minicells and present in the fraction of free proteins (A), and in the membrane fraction (B), after metrizamide density gradient equilibrium sedimentation of the minicell lysate obtained according to [19] and incubated with DNase (500 μg/ml, 10 mM $MgCl_2$, 60 min, 0°C). ^{14}C-Labelling of proteins synthesized in phage-infected minicells, their electrophoretic separation, detection by fluorography and identification was performed as in the legend to fig.1. The designation of protein peaks is the same as for fig.1.

λ*intc*-infected minicells were analyzed in this way, Int appeared exclusively in the membrane fraction (fig.3). We have proved that in this system membrane-binding of Int was also resistant to the action of 0.125 M KCl. The non-ionic detergent Triton X-100 (0.1%) caused partial elution, and the mixture of Brij-58 (0.5%) and deoxycholate (0.2%) caused complete elution of Int from the membrane, and its appearance in the free protein fraction (not shown).

Does Int bind to the membrane directly, or through λ DNA fragments protected against the action of DNase? In minicells, λ DNA directs the efficient, constitutive synthesis of Int, but it does not replicate [23]. This leads to a great surplus of Int in relation to λ DNA, and makes the model Int–DNA–membrane improbable for the majority of Int molecules. As salt at $\geqslant$2 M will dissociate most DNA binding proteins [25], Int ought to be eluted by 2 M KCl from the membrane, if it is bound through λ DNA. Since this is not the case, and because only detergents are effective in this respect, we presume, that Int binds directly to the membrane, and that hydrophobic interactions play a considerable role in this process.

Our results suggest, but do not prove, that membrane binding of Int occurs in vivo. This problem, as well as the possible role of cell membrane in the regulation of Int-mediated recombination need further studies.

Acknowledgements

We are grateful to Mrs Ewa Brzychcy for her excellent technical assistance. This work was supported by the Polish Academy of Sciences within project 09.7. M. Ż. was a Postgraduate Scholarship Fellow of the School of Medicine in Gdańsk.

References

[1] Nash, H. A. (1977) Curr. Top. Microbiol. Immunol. 78, 171–199.
[2] Weisberg, R. A., Gottesman, S. and Gottesman, M. E. (1977) in: Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R. eds) vol. 8, pp. 197–278, Plenum, New York.
[3] Miller, H. I., Kikuchi, A., Nash, H. A., Weisberg, R. A. and Friedman, D. I. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 1121–1126.
[4] Hendrix, R. W. (1971) in: The Bacteriophage Lambda (Hershey, A. D. ed) pp. 355–370, Cold Spring Harbor Laboratory, NY.
[5] Ausubel, F. (1974) Nature 247, 151–154.
[6] Nash, H. A. (1974) Nature 247, 543–545.
[7] Katzir, N., Oppenheim, A., Belfort, M. and Oppenheim, A. D. (1976) Virology 74, 324–331.
[8] Kotewicz, M., Chung, S., Takeda, Y. and Echols (1977) Proc. Natl. Acad. Sci. USA 74, 1511–1515.
[9] Kikuchi, Y. and Nash, H. A. (1978) J. Biol. Chem. 253, 7149–7157.
[10] Kotewicz, M., Grzesiuk, E., Courschesne, W., Fischer, R. and Echols, H. (1980) J. Biol. Chem. in press.
[11] Reeve, J. N. (1977) Mol. Gen. Genet. 158, 73–79.
[12] Lipińska, B., Skórko, R. and Taylor, K. (1978) Biochem. Biophys. Res. Commun. 82, 1202–1206.
[13] Shimada, K. and Campbell, A. (1974) Virology 60, 157–165.
[14] Grzesiuk, E. and Taylor, K. (1977) Virology 83, 329–336.
[15] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[16] Lipińska, B. (1979) PhD Thesis, University of Gdańsk.
[17] Lipińska, B., Podhajska, A. and Taylor, K. (1980) Biochem. Biophys. Res. Commun. 92, 120–126.
[18] Tait, R. C. and Boyer, H. W. (1978) Cell 13, 73–81.
[19] Żylicz, M. and Taylor, K. (1980) in preparation.
[20] Abraham, J., Mascarenhas, D., Fischer, R., Benedik, M., Campbell, A. and Echols, H. (1980) Proc. Natl. Acad. Sci. USA in press.
[21] Oppenheim, A., Katzir, N. and Oppenheim, A. B. (1977) Virology 79, 437–441.
[22] Oppenheim, A. and Oppenheim, A. B. (1978) Mol. Gen. Genet. 165, 39–46.
[23] Witkiewicz, H. and Taylor, K. (1979) Biochim. Biophys. Acta 564, 31–36.
[24] Clewell, D. B. and Helinski, D. R. (1970) Biochemistry 9, 4428–4440.
[25] Alberts, B. M., Amodio, F. J., Jenkins, M., Girtman, E. D. and Ferris, F. L. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 289–305.