Characterization of antibodies to chicken riboflavin carrier protein

Immunoneutralizing ability of antibodies to a sequence-specific region of the protein

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The properties of antibodies generated in rabbits against native riboflavin carrier protein (cRCP), riboflavin carrier protein that had been denatured/renatured by SDS treatment (SDS-RCP) or disulphide-bond-reduced then *S*-carboxymethylated (Carb-RCP) were studied. SDS-RCP could displace native RCP in radioimmunoassay (r.i.a.), whereas Carb-RCP could not. By using antibodies raised in five different rabbits against native cRCP, ¹²⁵I-labelled Carb-RCP could bind between 0 and 30% of the native antibodies. Antibodies raised against native RCP appear to be largely directed towards specific conformational determinants of RCP. Carb-RCP displaced native RCP in an r.i.a. using antibodies raised against SDS-RCP. SDS denaturation presumably unmasks cryptic epitopes in native RCP. Carb-RCP was a weak immunogen and elicited, presumably, antibodies to sequential epitope/epitopes. When injected into pregnant mice the antibodies caused neutralization of RCP, leading to termination of pregnancy, indicating highly conserved sequential epitopes in chicken and rodent RCP. Antibodies raised against Carb-RCP or native RCP reacted with CNBr fragments of native RCP, further confirming the presence of sequence-specific antibodies elicited by Carb-RCP.

INTRODUCTION

Riboflavin carrier protein (RCP) is a phosphoglycoprotein of M_r 34000 ± 2000. It was first isolated from hen's-egg white (Rhodes *et al.*, 1959) and yolk (Ostrowski *et al.*, 1968); it is a non-enzymic flavoprotein and binds riboflavin preferentially, with an affinity of $(1-5) \times 10^8 \text{ M}^{-1}$ (Murthy *et al.*, 1976; Becvar & Palmer, 1982). Recent studies have demonstrated the existence of a similar protein in rodents (Muniyappa & Adiga, 1980; Natraj *et al.*, 1987), cattle (Merrill *et al.*, 1979) and human (Murty & Adiga, 1982a; Natraj *et al.*, 1987) and monkeys (U. Natraj & S. D. Kholkute, unpublished work). The complete amino acid sequence of cRCP has been reported (Hamazume *et al.*, 1984). Monoclonal antibodies to cRCP have also been produced, and one epitope appears to be highly conserved in RCPs from various sources (Karande *et al.*, 1987). Immunoneutralization can be used as a specific assay for testing the functional property of RCP. In the absence of sequence data for RCPs from different sources, the antibodies specific for different epitopes of cRCP could be used for immunoneutralization of the RCP in different species. This approach would help identify the conserved epitopes of RCP. In the present study the properties of antibodies generated against cRCP, SDS-RCP and Carb-RCP will be discussed.

EXPERIMENTAL

Materials

Chemicals. DEAE-cellulose (0.9 mequiv./g), CM-cellulose (0.71 mequiv./g), Sephadex gels, dithiothreitol (DTT), 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) and SDS were from Sigma. The Vectastain ABC Kit was from Vector Laboratories, Burlingame, CA, U.S.A. All other chemicals were of analytical grade.

Animals. Inbred adult Swiss mice (10–20 weeks old) were from the animal colony of this Institute and were fed pelleted food (Hindustan Lever Products, Bombay, India) and water *ad libitum*. Female mice were mated with fertile adult males, and the day on which a vaginal plug was observed was designated as day 1 of pregnancy. For all the experiments described herein, day 11 of pregnancy was chosen.

Methods

Isolation and purification of chicken RCP from hen's-egg white. The method described by Murthy & Adiga (1977) was employed for isolation and purification of RCP. By this procedure, RCP free of bound riboflavin (the apoprotein) is obtained. The methodology used for examining the homogeneity of RCP was as reported previously (Natraj *et al.*, 1987).

Denaturation/renaturation of cRCP. The purified cRCP (5 mg) was treated with 1% (w/v) SDS in phosphate-buffered saline (PBS) for 2 h at 56 °C and dialysed against PBS and concentrated by freeze-drying to give SDS-RCP.

Abbreviations used: RCP, riboflavin carrier protein; cRCP, native RCP; SDS-RCP, RCP denatured/renatured by SDS treatment; Carb-RCP, disulphide-bond-reduced then *S*-carboxymethylated RCP; r.i.a., radioimmunoassay; DTT, dithiothreitol; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); PBS, phosphate-buffered saline (10 mM-sodium phosphate/0.15 M-NaCl, pH 7.0); PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin.

Modification of disulphide bonds and S-carboxymethylation. Reduction of disulphide bonds of cRCP was carried out essentially as described previously (Murthy *et al.*, 1976). The number of disulphide bonds reduced was estimated by using Ellman's reagent (Nbs₂; Habeeb, 1972). Carboxymethylation of the reduced protein was carried out by an established procedure (Gurd, 1967). In brief, 5 mg of RCP in 10 M-urea/100 mM-Tris, pH 8.5, was reduced with 100 mM-DTT for 2 and 18 h at 56 °C, and the reaction was terminated by alkylation with the addition of 1 M-iodoacetic acid, pH 8.0 (20-fold molar excess). The protein (Carb-RCP) was then dialysed extensively against water and freeze-dried. In the presence of 10 M-urea, six and seven disulphide bonds were reduced at 2 and 18 h respectively. Hence in all the studies only Carb-RCP reduced for 2 h was used.

Amino acid analysis. The number of disulphide bonds reduced was confirmed by the estimation of carboxymethylcysteine formed. The reduced and alkylated protein was hydrolysed in evacuated sealed tubes with 6 M-HCl for 24 h. The amino acid analysis was performed with a Beckman model 119 CL amino acid analyser.

Immunological techniques. Antibodies to native RCP ($n = 5$), SDS-RCP ($n = 2$) and Carb-RCP ($n = 3$) were generated in albino rabbits. The immunization dose and schedule followed were as follows. A 200 µg sample of the protein emulsified with an equal volume of Freund's complete adjuvant was injected subdermally at multiple sites once every 15 days for 2 months. The presence of circulating antibodies was tested by the Ouchterlony double-diffusion test or by direct binding to ¹²⁵I-Carb-RCP. After this the rabbits received a single intravenous booster dose of 200 µg of the protein in 0.9% NaCl. Rabbits were bled 10 days after the booster once every 2 weeks, and the serum from each bleeding was stored separately at -20 °C.

Radioimmunoassay for cRCP. The radioimmunoassay was carried out as detailed elsewhere (Natraj *et al.*, 1987). RCP and Carb-RCP were iodinated at room temperature by using the chloramine-T method, and the separation of labelled protein from free iodine was achieved by gel filtration through Sephadex G-75.

Immunoreactivity of SDS-RCP and Carb-RCP. The ability of modified RCP to displace the binding of cRCP was tested in the homologous-cRCP r.i.a. For displacement studies, 0.1–40 ng of cRCP, 0.1–500 ng of SDS-RCP or 0.1–500 ng of Carb-RCP was used.

Direct binding of ¹²⁵I-labelled cRCP and ¹²⁵I-labelled Carb-RCP. ¹²⁵I-RCP (30000 c.p.m.) or ¹²⁵I-Carb-RCP (30000 c.p.m.) were incubated with 0.1 ml of antiserum at several dilutions (1:100, 1:1000 and 1:10000) at 4 °C for 24 h. The total assay volume was 0.5 ml. At the end of the incubation, 0.1 ml each of 1:100-diluted normal rabbit serum and 1:10-diluted goat antiserum to rabbit immunoglobulin previously titrated for its equivalence point were added and further incubated for 48 h. The tubes were then centrifuged for 30 min at 5000 g and the radioactivity in the immunoprecipitate was counted. The non-specific binding was approx. 3% of the total radioactivity added.

Riboflavin-apoprotein interaction. The fluorescence of riboflavin is completely quenched on interaction with the apoprotein. The ability of modified apoprotein to interact with riboflavin was monitored spectrofluorimetrically (Murthy *et al.*, 1976).

Ability of antibodies to RCP and modified RCP to terminate pregnancy in mice. Day-11-pregnant mice were injected with 0.1–0.5 ml of de complemented antiserum to Carb-RCP, SDS-RCP and cRCP. The animals were killed 24 h later, and the implantation sites examined for the presence of live fetuses.

CNBr fragments. Apoprotein (5 mg) was subjected to CNBr (50 mg) cleavage in 70% (w/v) formic acid for 4 h at 4 °C. The reaction was terminated by dilution with water and freezing. Excess CNBr was removed by repeated freeze-drying (Gross, 1967). CNBr-cleaved RCP was subjected to SDS/PAGE on 12.5%-(w/v)-acrylamide gels (Laemmli, 1970) as well as on 8–25% gradient gels without urea under reducing conditions (Fling & Gregerson, 1986).

Immunoblotting: enzyme-linked immunoelectrotransfer blot. Apoprotein and CNBr-cleaved fragments were subjected to SDS/PAGE on 12.5%-acrylamide gels (Laemmli, 1970). Immediately after electrophoresis the gel was equilibrated in the transfer buffer (0.025 M-Tris/0.193 M-glycine/20% (v/v) methanol, pH 8.3) for 30 min, and then the protein was electrophoretically transferred to a nitrocellulose filter overnight at 30 V (Towbin *et al.*, 1979) by using a Bio-Rad Transblot cell. Before incubation with antibodies, the non-specific sites were blocked by incubation with 5% BSA for 60 min. This was followed by incubation with a 1:1000 dilution of antiserum to native cRCP or a 1:250 dilution of antibody to Carb-RCP overnight. The paper was then rinsed three times with PBS/Tween (0.01 M-Na₂HPO₄/NaH₂PO₄/0.15 M-NaCl, pH 7.2, with 0.3% Tween 20). The nitrocellulose paper was exposed to biotinylated goat anti-rabbit IgG for 60 min and washed once again and incubated with preformed avidin-biotinylated horseradish peroxidase complex reagent for 2 h. After washing thoroughly with PBS/Tween, the paper was then exposed to the substrate solution (5 mg of 3,3'-diaminobenzidine was dissolved in freshly made 10 ml of PBS containing 0.01% H₂O₂) and the antigen-antibody reaction was then photographed.

RESULTS

Effect of modification of RCP on riboflavin binding and immunoreactivity

Carb-RCP was unable to bind riboflavin, as monitored spectrofluorimetrically. Binding of riboflavin apoprotein is inhibited in the presence of 1% SDS (Murthy *et al.*, 1976.) However, on renaturation, apoprotein binds to riboflavin (Fig. 1).

The ability of modified RCP to displace cRCP in the r.i.a. is depicted in Fig. 2. Carb-RCP failed to displace native protein, even at a 500-fold excess. However, SDS-RCP was effective in displacing the cRCP.

Binding of ¹²⁵I-RCP and ¹²⁵I Carb-RCP to antibodies to cRCP

Fig. 3 depicts the ability of Carb-RCP to bind

Fig. 1. Spectrofluorimetric titration of riboflavin with various amounts of apoprotein (●), SDS-RCP (○) and Carb-RCP (▲)

The interaction of riboflavin with cRCP, Carb-RCP and SDS-RCP was monitored spectrofluorimetrically on an Amincon-Bowman spectrophotofluorimeter. The riboflavin was excited at 370 nm and the emission was analysed at 520 nm. Various amounts of cRCP, SDS-RCP and Carb-RCP were added to 3.0 ml of riboflavin (4×10^{-7} M) in 0.1 M-Tris, pH 7.4. For further details, see Murthy *et al.* (1976).

Fig. 2. Immunoreactivity of SDS-RCP and Carb-RCP with antiserum to cRCP

The displacement of 125 I-labelled cRCP by its antiserum (1:20000) in the presence of cRCP (○), SDS-RCP (▲) and Carb-RCP (□) is shown. The r.i.a. was performed as detailed by Natraj *et al.* (1987).

antibodies to native RCP at various dilutions. Of the several rabbits immunized, only few produced antibodies which did not bind 125 I-Carb-RCP. Rabbits 1704, 1803 and 1667, immunized with native RCP, generated

Fig. 3. Binding of 125 I-RCP and 125 I-Carb-RCP to antibodies to native RCP

□, 125 I-RCP binding; ■, 125 I-Carb-RCP binding; a, b and c represent initial antibody concentrations of 1:100, 1:1000 and 1:10000 respectively. The numbers on the abscissa are those assigned to individual rabbits.

Fig. 4. Immunoreactivity of Carb-RCP towards the antiserum to SDS-RCP

The displacement of 125 I-labelled cRCP in the presence of cRCP (▲) and Carb-RCP (●) is shown. The dilution of the antiserum was 1:20000. The other assay conditions are the same as those given in Fig. 2.

antibodies that bound native RCP and also bound 10–30 % of 125 -Carb RCP.

Ability of Carb-RCP to interact with antibodies generated against SDS-RCP

In the cRCP r.i.a. system a 50% inhibition was obtained with 0.37 ng of native protein, whereas 100 ng of Carb-RCP was required to inhibit 50 % of binding (Fig. 4). However, the displacement curve was parallel with that obtained with the native protein.

Immunological properties of antibodies generated against Carb-RCP

Carb-RCP appeared to be a poor immunogen in all the three rabbits tested. No precipitin lines were obtained when the reaction was analysed by the Ouchterlony gel-diffusion technique. Irrespective of whether the antibody was raised against Carb-RCP, native RCP or SDS-RCP,

Table 1. Effect of administration of antiserum to cRCP, SDS-RCP and Carb-RCP to pregnant mice

The pregnant mice were injected intraperitoneally with normal rabbit serum (0.5 ml), anti-cRCP (0.1 ml), anti-SDS-RCP (0.1 ml) and anti-Carb-RCP (0.2 or 0.3 ml). The sera were incubated at 56 °C for 30 min to inactivate the complement. After a single injection on day 11 of pregnancy, all the animals were killed 24 h later.

Treatment	Implantation sites	Remarks
Normal rabbit serum	8 ± 1	Normal fetus
Antiserum to: cRCP	8 ± 1	Vaginal bleeding by 6 h; dead fetus
SDS-cRCP	8 ± 1	Vaginal bleeding by 6 h; dead fetus
Carb-RCP	8 ± 1	Incomplete (six resorbed fetuses, two normal)
Carb-RCP (0.3 ml)	8 ± 1	Complete resorption

the binding of ^{125}I Carb-RCP was of the same order, that is, approx. 10–30 %.

Effect of the administration of antisera to SDS-RCP and Carb-RCP in pregnant mice

To study the ability of antisera to Carb-RCP and to

SDS-RCP to terminate pregnancy in day-11-pregnant mice, several doses of the antiserum were administered. The results indicated that a single injection of 0.3 ml of antiserum to Carb-RCP and 0.1 ml of antiserum to SDS-RCP resulted in complete resorption of the fetuses. A single dose of 0.1 ml of antiserum to native RCP also resulted in 100 % resorption. At a dose of 0.2 ml of antiserum to Carb-RCP, partial resorption of fetuses was observed (Table 1).

Binding of anti-cRCP and anti-Carb-RCP antibodies to CNBr-cleaved fragments.

There are seven methionine residues in cRCP. The largest fragment is 123 amino acids long (Hamazume *et al.*, 1984). Limited CNBr cleavage was carried out to generate fragments which could be probed with antibodies. When CNBr-cleaved cRCP was subjected to SDS/PAGE under reducing conditions (Fig. 5a), two or three fragments, with M_r values of 24000, 20000 and 18000, were obtained. However, on 8–25 % gradient gels (Fig. 5b), six fragments were obtained, of which at least two fragments had M_r values of less than 3400 (Fig. 5b). The fragments were transferred to nitrocellulose and then probed with antibodies to native RCP as well as to Carb-RCP. The antibody reaction was revealed with ABC stain (Fig. 6). Antiserum to native RCP binds to both CNBr fragments. However, antiserum to Carb-RCP bound strongly to the CNBr fragments, but weakly, if at all, to native RCP. Lower- M_r fragments did not react with either of these antibodies.

Fig. 5. SDS/PAGE of RCP and CNBr-cleaved RCP

(a) SDS/PAGE (12.5 % gel): lane 1, RCP; lane 2, CNBr-cleaved RCP (25 μg); lane 3, SDS-treated standards. (b) SDS/PAGE (8–25 % gradient gel) without urea: lane 1, cRCP (25 μg); lanes 2 and 3, CNBr fragments generated on two different batches of cRCP (25 μg) (note two fragments below M_r 3500); lanes 4 and 5, SDS-treated standards; lane 6, insulin B-chain (M_r 3500). Standards used were BSA (M_r 66000), ovalbumin (45000), trypsinogen (24000), lactoglobulin (18400), lysozyme (14300) and insulin (B-chain 3500).

Fig. 6. Antigenic activity of electrophoretic replicates

CNBr-cleaved RCP (15 µg, lane 1) and cRCP (10 µg, lane 2) were allowed to react with (a) rabbit antiserum against cRCP (rabbit 1803; 1:1000) and (b) rabbit antiserum against Carb-RCP (1814, 1:250).

DISCUSSION

The aim of the present study was to characterize the antibodies generated against the native and the modified protein to gain an insight into the relative importance of determinants in the sequence and those present in the non-continuous sequence, i.e. made up of amino acids from different parts of the polypeptide brought together by chain-folding 'assembled determinants' to induce neutralizing immunity.

It has been reported that there are nine disulphide bonds in cRCP (Hamazume *et al.*, 1987). Our previous studies (Murthy *et al.*, 1976) had indicated that reduction of disulphide bonds leads to a loss in the riboflavin binding. This was confirmed in the present study also (Fig. 1). Here we provide evidence that the conformational changes associated with the loss in the riboflavin binding is also associated with the loss in its ability to interact with antibodies to native RCP in an r.i.a. system (Fig. 2). The low binding of ^{125}I -Carb-RCP clearly indicates that most of the antibodies to RCP are directed towards conformational determinants. These results appear to follow the general trend set by other globular proteins (Benjamin *et al.*, 1984). Thus proteins rich in disulphide bonds elicit antibodies which are specific to the conformation of the protein, as in the case of lysozyme (Arnon *et al.*, 1971), ribonuclease (Brown *et al.*, 1959) and human choriongonadotropin (Ghai

et al., 1980). In the case of staphylococcal nuclease (Reichlin, 1975) and myoglobin (Atassi & Lee, 1978), which do not have disulphide bonds, a significant fraction of the antigenicity resides in sequential determinants. In the case of actin, carboxymethylation led to an increase in the antigenicity, and such a modified protein produced antibodies to the *N*-terminal segment (Benyamin & Boyer, 1986).

Carb-RCP is a poor immunogen and elicited a weak antibody response. This is presumably owing to there being very few epitope/epitopes available for antibody production. The antiserum to Carb-RCP, although it binds only to the extent of 10–30 % to cRCP, was able to immunoneutralize rodent RCP and cause resorption of the fetus and termination of pregnancy. This indicates that (1) an epitope/epitopes present in the continuous (sequence) segment of chicken RCP is present in the rodent RCP and (2) antibodies against this sequence lead to neutralization of the native protein.

Treatment of RCP with 1 % SDS followed by dialysis does not alter its ability to displace cRCP in r.i.a., and antibodies raised against this antigen were able to immunoneutralize rodent RCP to the same extent as did the native protein. However, when such SDS-treated protein was used for immunization, unmasking of certain determinants was evident. Thus antibodies raised against this antigen bind with greater avidity to the unmasked determinants. This determinant could well be a sequential rather than an assembled determinant; Carb-RCP contains predominantly such determinants, and hence is able to displace native RCP in an r.i.a. using antibodies raised against SDS-RCP but not in an r.i.a. using antibodies raised against native RCP (Fig. 4). The possible sequence/sequences recognized by antibodies to Carb-RCP and native RCP was analysed by limited CNBr cleavage. Antibodies generated against Carb-RCP and native RCP interacted with larger cleaved fragments. This is highly suggestive of sequence-specific epitope(s). Since antibodies to Carb-RCP are of the neutralizing type, it can be argued that one of the epitopes of RCP is present in this sequence. Acrophilic and hydrophilic profiles (Hopp, 1985) generated from the known sequence of RCP shows at least three epitopes (K. S. H. Iyer & K. Dhanasekharan, unpublished work). One epitope is present in the internal region and spans the major CNBr fragment. It would be interesting to examine whether a synthetic peptide of this sequence would induce neutralizing immunity.

In the absence of well-characterized RCPs from the other sources, the use of antibodies to map epitopes of cRCP and to examine its neutralizing ability in the rodent model should eventually lead to the determination of the sequence which is common to other species. From such studies it should be possible to synthesize a peptide that could generate antibodies to the conserved region of the protein.

In conclusion, these studies indicate that native RCP generates antibodies that are to a large extent conformational, and SDS treatment does result in unmasking of cryptic epitopes. Antibodies generated against unfolded protein (Carb-RCP) are to a sequence-specific epitope common to mouse RCP and cRCP.

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REFERENCES

- Arnon, R., Maron, E., Sela, M. & Anfinsen, C. B. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1450–1455
- Atassi, M. Z. & Lee, C. L. (1978) *Biochem. J.* **171**, 429–434
- Becvar, J. & Palmer, G. (1982) *J. Biol. Chem.* **257**, 5607–5617
- Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Reichlin, M., Sercarz, E. E., Smith-Gill, J., Todd, P. K. & Wilson, A. C. (1984) *Annu. Rev. Immunol.* **2**, 67–101
- Benyamin, C. R. & Boyer, M. (1986) *J. Immunol. Methods* **86**, 21–29
- Brown, R. K., Delaney, R., Leuine, L. & Van Vunakis, H. (1959) *J. Biol. Chem.* **234**, 2043–2049
- Fling, S. P. & Gregerson, D. S. (1986) *Anal. Biochem.* **155**, 83–88
- Ghai, R. D., Mise, T., Pandian, M. R. & Bahl, O. P. (1980) *Endocrinology (Baltimore)* **107**, 1556–1563
- Gross, E. (1967) *Methods Enzymol.* **11**, 238–255
- Gurd, F. R. N. (1967) *Methods Enzymol.* **11**, 532–541
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Hamazume, Y., Mega, T. & Ikenaka, T. (1984) *J. Biochem. (Tokyo)* **95**, 1633–1644
- Hamazume, Y., Mega, T. & Ikenaka, T. (1987) *J. Biochem. (Tokyo)* **101**, 217–233
- Hopp, T. P. (1985) in *Synthetic Peptides in Biology and Medicine* (Alitalo, K., Partanen, D. & Vaheri, A., eds.), pp. 3–12, Elsevier Science Publishers, Amsterdam, New York and Oxford
- Karande, A. A., Visweswariah, S. S. & Adiga, P. R. (1987) *J. Immunol. Methods* **99**, 173–177
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Merrill, A. H., Jr., Prochlich, J. A. & McCormic, B. (1979) *J. Biol. Chem.* **254**, 9362–9364
- Muniyappa, K. M. & Adiga, P. R. (1980) *FEBS Lett.* **110**, 209–212
- Murthy, U. S. & Adiga, P. R. (1977) *Indian J. Biochem. Biophys.* **14**, 118–124
- Murthy, U. S., Podder, S. K. & Adiga, P. R. (1976) *Biochem. Biophys. Acta* **434**, 68–81
- Murty, C. V. R. & Adiga, P. R. (1982a) *Biochem. Int.* **5**, 289–296
- Murty, C. V. R. & Adiga, P. R. (1982b) *Science* **216**, 191–193
- Natraj, U., Asok Kumar, R. & Kadam, P. (1987) *Biol. Reprod.* **36**, 677–685
- Natraj, U., George, S. & Kadam, P. (1988) *J. Reprod. Immunol.*, in the press
- Ostrowski, W., Zak, Z. & Kauczy, K. A. (1968) *Acta Biochem. Pol.* **15**, 241–259
- Reichlin, M. (1975) *Adv. Immunol.* **20**, 71–123
- Rhodes, M. B., Bennett, N. & Feeney, R. E. (1959) *J. Biol. Chem.* **234**, 2054–2060
- Towbin, H., Staehlein, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354

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