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ENDOGENOUS AND CHOLERA TOXIN-CATALYZED ADP-RIBOSYLATION OF A PLASMA MEMBRANE PROTEIN OF RL-PR-C CLONED RAT HEPATOCYTES *

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Summary

Cholera toxin catalyzed the ADP-ribosylation of a single plasma membrane protein (M_r 55 000) of both RL-PR-C rat hepatocytes and purified rat liver plasma membranes. Labeling of this protein from nicotinamide [2,8-^{3}H]adenine dinucleotide was competitively inhibited by free arginine, but by no other amino acid tested, including lysine.

The same protein was ADP-ribosylated from NAD^+ endogenously, i.e., in the absence of toxin. This process was, however, not competitively inhibited by added arginine nor by any other amino acid tested, including lysine.

Free ADP-ribose, even in 50-fold molar excess over the nicotinamide [2,8-^{3}H]adenine dinucleotide substrate, did not reduce (by isotope dilution) the endogenous or cholera toxin-catalyzed labeling of the 55 000 dalton membrane protein. It is likely, therefore, that hepatocyte plasma membranes contain an ADP-ribosyltransferase, with a mechanism similar to that of the A subunit of cholera toxin, in that both transfer ADP-ribose to the same membrane protein and in that neither apparently produce free ADP-ribose as an intermediate. It is also clear that the acceptor residue in the 55 000 dalton protein is different for each process.

Cholera toxin-catalyzed and endogenous transfer of ADP-ribose to the hepa-

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Abbreviations: SDS, sodium dodecyl sulfate; PRO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-yl-2)benzene; Hepes, *N*,2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tocyte plasma membrane protein, in contrast to a pigeon erythrocyte system, required no cytosolic factors.

The results indicate that ADP-ribosylation in cloned differentiated rat hepatocytes differs from that in pigeon erythrocytes in that the acceptor protein is larger (55 000 compared to 42 000 daltons), cytosolic factors are not required, and transfer of ADP-ribose to the acceptor protein occurs endogenously.

Introduction

It has been postulated that the activation of adenylate cyclase in eukaryotic cells by cholera toxin [1—3] is mediated by ADP-ribosyltransferase activity [4,5] intrinsic to the A_1 fragment of the toxin [6], and that ADP-ribosylation of some membrane component is integral to the activation process. A 42 000 dalton membrane acceptor of cholera toxin-catalyzed ADP-ribosylation has been identified in pigeon erythrocytes [7], hyman red bood cells [8], and in S49 lymphoma and HTC4 rat hepatoma cells [9]. In avian erythrocytes, this acceptor appears to be responsible for guanine nucleotide and fluoride sensitivity of adenylate cyclase [10]. In a phenotypic variant of S49 cells *cyc*$^-$ [11], the adenylate cyclase of which is not regulated by guanine nucleotides, F^- or hormones [12], no cholera toxin-catalyzed ADP-ribosylation of membrane components occurs [9]. These observations suggested that the site of cholera toxin modification is the guanine nucleotide regulatory component that is associated with adenylate cyclase [13]. S49 lymphoma and HTC4 hepatoma cell membranes appear also to contain acceptors for ADP-ribosylation other than the 42 000 dalton protein [9], and it has been suggested [14,15] that these additional components could also be involved in adenylate cyclase regulatory phenomena in mammalian cells.

We have been studying a chromosomally and culturally stable cloned line of rat hepatocytes [16], RL-PR-C, which retains many differentiated characteristics, including adenylate cyclases that are responsive to glucagon [17], catecholamines, guanine nucleotides, and F^- [18]. Further, these cells respond to cholera toxin with the incorporation of ADP-ribose from NAD^+ into membrane protein, followed by marked activation of adenylate cyclase [19]. The present study demonstrates that the sole non-nuclear acceptor for ADP-ribosylation in RL-PR-C hepatocytes is a membrane protein of M_r 55 000 and that endogenous ADP-ribosylation of the same acceptor is catalyzed by a membrane, not cytosolic, enzyme.

Experimental procedures

Cell culture. RL-PR-C hepatocytes [16] were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum, as described [20]. Confluent monolayers were fed 16 h prior to assay, and 4000 × *g* membrane fractions were prepared as previously described [19].

Materials. Nicotinamide [2,8-^{3}H]adenine dinucleotide was obtained from New England Nuclear and *carbonyl*[^{14}C]NAD^+ from Amersham. Cholera toxin was obtained from Schwarz-Mann and reconstituted as directed. L-amino acids and analogues were from Calbiochem.

ADP-ribosylation. Except where indicated, the final concentrations in the reaction mix were 50 mM Tris-HCl (pH 7.5), 100 μM GTP, 2 mM dithiothreitol, 10 μM labeled NAD^+ (3500 cpm/pmol) and 1 μg/ml cholera toxin, in a final volume of 100 μl. The reaction was initiated by the addition of 200 μg membrane protein, and, after 10 min at 37°C, ended by the addition of ice-cold Tris-HCl buffer (pH 7.5) and the centrifugation of the particulate fraction in a microfuge. The pellet was washed once with Tris-HCl buffer and solubilized in 26.7 mM H_2SO_4/54.1 mM Tris base/1% (w/v) SDS/1% (v/v) β-mercaptoethanol, pH 6.5, at 37°C.

SDS-polyacrylamide gel electrophoresis. The procedure was essentially that of Neville and Glossman [21]. The running gel was 13% acrylamide (0.8% bisacrylamide) in 0.0308 N HCl/0.424 M Tris base/0.1% SDS, pH 9.18. The stacking gel was 3.2% acrylamide (6.25% bisacrylamide) in 26.7 mM H_2SO_4/54.1 mM Tris/0.1% SDS, pH 6.1. The upper tray buffer was 40 mM boric acid/41 mM Tris/0.1% SDS, pH 8.64, and the lower tray buffer 0.0308 N HCl/0.424 mM Tris base, pH 9.18. Gels (150 mm) were stacked at 0.5 mA/ gel and run at 1.5 mA/gel. Following electrophoresis, gels were stained with 0.1% Coomassie Blue in 50% methanol in 10% acetic acid and destained in 5% methanol in 10% acetic acid. Gels were sliced into 1 mm slices, heated overnight in Protosol · H_2O (9 : 1, v/v), and counted in 0.8% PPO/0.02% POPOP in toluene.

Adenylate cyclase. Enzyme activity was assayed according to the method of Salomon et al. [22]. Final concentrations were as follows: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM cyclic AMP, 1 mg/ml bovine serum albumin, 10 units creatine phosphokinase, 20 mM phosphocreatine, 0.5 mM [α-^{32}P]ATP (60 Ci/mol), 2 mM dithiothreitol and additions as indicated.

Protein determination was by the method of Lowry et al. [23].

Results and Discussion

The activation of adenylate cyclase in RL-PR-C hepatocyte membranes by cholera toxin required NAD^+; no other nicotinic or adenine nucleotide could substitute (Table I). The decrease in basal adenylate cyclase activity caused by adenine nucleotides may be related to the adenosine inhibitory regulatory site postulated by Londos and Preston [24] on the basis of studies with purified rat liver plasma membranes. The inability of NADH even partially to substitute for NAD^+ is likely due to the absence of the enzymes required to oxidize NADH in the plasma membrane preparation employed.

We have previously reported [19] that cholera toxin catalyzed the incorporation of ADP-ribose into trichloroacetic-acid-insoluble material of a RL-PR-C membrane preparation. When the labeled components of the crude membrane preparation were separated by polyacrylamide gel electrophoresis, the labeling pattern shown in Fig. 1 was obtained. The bands located at the top of the gel probably represent the incorporation of labeled ADP-ribose into polymers covalently linked to various nuclear chromatin acceptors [25]. This area of the gel stained heavily with ethidium bromide, and it is known that DNA does not dissociate and enter the gel under the conditions employed [26]. Furthermore, we found that incorporation of label into this area of the gel could be decreased by thymidine, which is known to inhibit nuclear poly(ADP-Rib)

TABLE I

EFFECTS OF NICOTINIC AND ADENINE NUCLEOTIDES ON BASAL AND CHOLERA TOXIN-ACTIVATED ADENYLATE CYCLASE ACTIVITIES IN RL-PR-C HEPATOCYTES

Adenylate cyclase was assayed in membrane preparations, with additions as noted in the table. All additions were at 10 mM except for NAD^+ (1 mM). Cholera toxin was used at 10 μg/ml. The values shown are means ± S.D. for triplicate determinations.

Additions	Adenylate cyclase activity (pmol cyclic AMP/mg protein per 10 min)		
	Basal	+ Cholera toxin	Change (%)
None	547 ± 105	638 ± 68	17
NAD^+	600 ± 110	890 ± 50	49
NADH	589 ± 41	583 ± 122	—
NADP	40 ± 2	27 ± 5	—
Nicotinic acid	576 ± 5	559 ± 15	—
Nicotinamide	52 ± 24	58 ± 22	—
Adenine	82 ± 29	68 ± 17	—
Adenosine	172 ± 24	186 ± 22	—
AMP	77 ± 9	90 ± 4	16
ADP	23 ± 9	20 ± 4	—
ADP-ribose	41 ± 17	44 ± 9	—
D-Ribose 5-phosphate	379 ± 47	393 ± 34	—

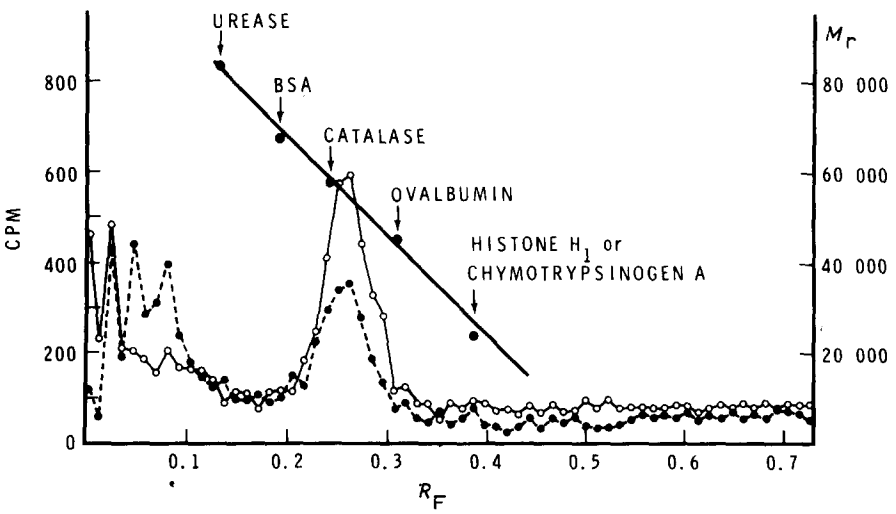

Fig. 1. SDS-polyacrylamide gel electrophoresis of membrane proteins. Monolayers of RL-PR-C hepatocytes were washed twice with phosphate-buffered saline, and the cells scraped from the flask with a rubber policeman. Cells were homogenized in 1 mM $NaHCO_3$, and a 4000 × *g* membrane fraction isolated [19]. Aliquots (200 μg protein) of membranes were ADP-ribosylated in the absence (●———●) or presence (○———○) of 1 μg/ml cholera toxin. Membrane proteins were solubilized and separated by gel electrophoresis, and the radioactivity in 1 mm gel slices determined. BSA, bovine serum albumin.

polymerase activity [27]. No significant ADP-ribosylation of histones, which did enter the gel, was observed.

Other than this high molecular weight chromatin-associated radioactivity, only a single band of radioactivity, corresponding to an M_r of approximately 55 000, was found in experiments with crude RL-PR-C hepatocyte membrane preparations (Fig. 1) as well as in purified rat liver plasma membranes (not shown). There was no radioactivity located at the top of the gel when nuclei-free purified rat liver plasma membranes [28] were labeled, supporting the view that such material was of nuclear origin in crude RL-PR-C hepatocyte membrane preparations (Fig. 1). The single 55 000 dalton ADP-ribose acceptor of RL-PR-C hepatocyte and rat liver plasma membranes is in contrast to the 42 000 dalton acceptor found in avian erythrocytes (Ref. 7 and our unpublished results) and in several mammalian cells. For example, Kaslow et al. [8] have identified two ADP-ribose acceptors in S49 lymphoma cells, one of M_r 42 000 (similar to that of avian erythrocytes) and the other a doublet of M_r 53 000—54 000 (similar to that of RL-PR-C cells). The authors suggest that the 53 000—54 000 dalton protein could be a precursor of the smaller acceptor or play a role in processes not exhibited by membranes lacking this larger component, e.g., agonist-specific refractoriness [14] or guanyl nucleotide regulation of receptor affinity [15]. The absence of a detectable 42 000 dalton acceptor component in both RL-PR-C cells and purified rat liver plasma membranes would, however, argue against a precursor role for the 55 000 dalton protein in this tissue.

In both purified rat liver plasma membranes and crude membrane preparations of RL-PR-C hepatocytes. ADP-ribose from nicotinamide [2,8-^{3}H]adenine dinucleotide was incorporated into the 55 000 dalton band even in the absence of cholera toxin (Fig. 1). The extents of both endogenous and cholera toxin-catalyzed incorporation were proportional to the concentration of NAD^+, while that due to cholera toxin was also proportional to toxin concentration, reaching a maximum at 10 ng/ml in the presence of 10 μM NAD^+. There was no incorporation of radioactivity into any area of the gel, in the presence or absence of cholera toxin, if [^{3}H]adenosine or [*carbonyl*-^{14}C]NAD^+ were substituted for nicotinamide [^{3}H]adenine dinucleotide. Futher, neither adenine nor adenosine (100 μM) inhibited the incorporation of radioactive ADP-ribose from nicotinamide [^{3}H]adenine dinucleotide. These results reveal that NAD^+, and not its catabolites, serves as the substrate for ADP-ribosylation.

Free ADP-ribose has been shown to form covalently bound adducts with a variety of peptides and proteins by the formation of Schiff bases with lysine residues [29]. It is unlikely that such a reaction occurs under conditions employed in the present studies since (i) added lysine had no effect on radioactive labeling of the 55 000 dalton protein (Table II), (ii) histones, reported to be good acceptors for ADP-ribose by this nonenzymatic mechanism [29], were not labeled (Fig. 1), and (iii) only one, rather than multiple, membrane proteins were ADP-ribosylated in the present system.

Endogenous transfer of ADP-ribose to a specific membrane protein has not been reported previously. Moss and Vaughan [30] have isolated a cytosolic protein from avian erythrocytes with ADP-ribosyltransferase activity similar to that of cholera toxin [31]. The enzyme transferred ADP-ribose to many pro-

TABLE II

EFFECT OF EXOGENOUS AMINO ACIDS AND ADP-RIBOSE ON ADP-RIBOSYLATION OF A HEPATOCYTE MEMBRANE PROTEIN

RL-PR-C hepatocyte membranes were ADP-ribosylated from nicotinamide [2,8-^{3}H]adenine dinucleotide and the membrane proteins separated electrophoretically. The counts incorporated into the 55 000 dalton band, corrected for background, were determined. The last column shows the amount of cholera toxin-catalyzed ADP-ribosylation. Values represent pmol ADP-ribose incorporated/mg membrane protein.

Additions	Endogenous	Endogenous + cholera toxin	Cholera toxin
Control	5.46	6.70	1.24
L-Lysine, 5.0 mM	5.82	7.11	1.29
Control	1.93	3.43	1.50
Agmatine, 5.0 mM	2.61	1.98	0
L-Ornithine, 5.0 mM	1.74	3.12	1.38
L-Citrulline, 5.0 mM	2.39	4.45	2.06
Control	6.48	8.99	2.51
L-Arginine			
1.0 mM	5.31	5.17	0
5.0 mM	5.15	4.49	0
ADP-ribose			
0.5 mM	5.99	10.02	4.03
1 mM	4.35	7.57	3.22
5 mM	1.52	2.49	0.97

teins, and it activated heterologous adenylate cyclases. The authors proposed that such endogenous ADP-ribosylation may regulate adenylate cyclase activity, and that the transferase may be controlled by compartmentalization, covalent modification or other effector molecules [30]. The endogenous ADP-ribosyltransferase activities of RL-PR-C hepatocytes and rat liver plasma membranes seem distinct from this avian erythrocyte cytosolic enzyme. Endogenous ADP-ribosyltransferase activity in the present experiments was associated with the plasma membrane, not the cytosol (Fig. 1), and free arginine was ineffective as an alternate acceptor of the ADP-ribose moiety in hepatocytes, in contrast to the situation with the avian cytosolic enzyme [30].

Johnson et al. [9] noted that arginine, ADP-ribose and thymidine decreased the 'non-specific background' incorporation of labeled ADP-ribose from NAD^+ into S49 lymphoma cells to an extent which permitted the observation of an effect of cholera toxin on the process. Although the thymidine effect suggested that some of the background incorporation was due to nuclear poly(ADP-ribose) polymerase [27], the basis for the effects of ADP-ribose and arginine was not clear [9]. To define further the mechanism of ADP-ribosylations in the hepatocyte system and to identify the acceptor(s), we tested various compounds for their ability to decrease the incorporation of the ADP-ribose moiety of nicotinamide [2,8-^{3}H]adenine dinucleotide into the 55 000 dalton protein of RL-PR-C hepatocyte membrane (Table II). The variability of endogenous ADP-ribosylation between experiments may be due to varying amounts of endogenous NAD^+ still associated with the membrane. Arginine and agmatine (the decarboxyl analogue of arginine) inhibited toxin-catalyzed labeling. The effect of

arginine was not mimicked by lysine, ornithine or citrulline. These observations confirm those of others working with model systems, in which it was concluded that the toxin catalyzes the transfer of the ADP-ribose moiety of NAD^+ to the guanidino group of arginine [5,32].

The acceptor residue for endogenous ADP-ribosylation is not known. High concentrations of exogenous arginine failed significantly to reduce endogenous ADP-ribosylation (Table II). Similarly ineffective were lysine, agmatine, ornithine, citrulline (Table II), or aspartate, asparagine, glutamate, glutamine, serine, threonine, tyrosine and histidine. The acceptor residue may be a modified amino acid analogous to the modified histidine residue of elongation factor-2 that is ADP-ribosylated from NAD^+ by diptheria toxin [33].

Neither endogenous nor cholera toxin-catalyzed labeling of the 55 000 dalton acceptor from nicotinamide [^{3}H]adenine dinucleotide was decreased by free ADP-ribose in concentrations up to 50-fold greater than that of labeled NAD^+ (Table II). An effect of free ADP-ribose was, however, observed at concentrations of at least 1 mM (Table II), a concentration 100-times greater than that of labeled NAD^+ (10 μM). If free ADP-ribose served as the substrate for ADP-ribosylation, much lower concentrations of exogenous ADP-ribose would have been adequate to decrease radioactive labeling from labeled NAD^+ by isotope dilution. These observations support the view that both endogenous and cholera toxin-catalyzed ADP-ribosylation proceed by a sequential mechanism which involves the formation of a ternary complex of enzyme, NAD^+ and acceptor protein, followed by direct transfer of the ADP-ribosyl moiety to the acceptor [34]. Although free ADP-ribose does not accumulate as an intermediate during the course of either reaction, a very large excess of free ADP-ribose, e.g., 1 mM, may decrease ADP-ribosylation by limited exchange with enzyme-bound ADP-ribose. This effect of ADP-ribose seemed more pronounced in the endogenous reaction (Table II, final column). The reason for this is not clear, but may be due to the nature of the acceptor moiety that is ADP-ribosylated endogenously.

It is quite likely, nevertheless, that free ADP-ribose could be produced by liver membranes from NAD^+. Both crude hepatocyte and purified rat liver plasma membrane preparations actively hydrolyzed substrate *carbonyl*-[^{14}C]NAD^+ under standard ADP-ribosylating conditions, hydrolyzing 2.24 ± 0.18 and 3.14 ± 0.01 pmol NAD^+ per μg protein in 10 min, respectively. The amounts of ADP-ribose produced from 10 μM nicotinamide [2,8-^{3}H]adenine dinucleotide by glycohydrolase activity would not, however, be sufficient to interfere with the ADP-ribosylation process (Table II). Although cholera toxin itself has been shown to possess NAD^+ glycohydrolase activity [4,6], such activity was not detectable under the ADP-ribosylating conditions employed here.

Although kinetic experiments [19] suggested a cause and effect relationship between ADP-ribosylation of RL-PR-C hepatocytes and activation of adenylate cyclase, the wide differences in sensitivity of both processes to cholera toxin in hepatocytes make it difficult to prove conclusively such a relationship. While ADP-ribosylation of hepatocyte membranes was evident with as little as 10 ng/ml cholera toxin, much greater concentrations of the toxin (5 μg/ml) were required to observe an effect on adenylate cyclase activity. Similarly, 10 μM NAD^+ was sufficient to observe toxin-catalyzed ADP-ribosylation, but toxin

activation of adenylate cyclase required at least 1 mM NAD^+. It is possible that multiple arginine residues of the 55 000 dalton protein must be modified before an effect of cholera toxin on adenylate cyclase activity can be observed. Alternatively, some of the toxin-catalyzed ADP-ribosylation may be non-productive, if, for example, ADP-ribosylation of the 55 000 dalton protein leads to the activation of adenylate cyclase only if the protein is in close association with the catalytic moiety of the enzyme. Answers to these questions must await reconstitution of resolved components of the enzyme complex.

Enomoto and Gill [35] reported that, in pigeon erythrocytes, there was an absolute requirement for a cytosolic factor for cholera toxin-catalyzed ADP-ribosylation of cell membranes. No such requirement for homologous cytosol was observed in the present studies with hepatocytes (Table III). Similarly, cytosol was not required for either ADP-ribosylation or activation of adenylate cyclase by cholera toxin in S49 lymphoma and HTC4 hepatoma calls [9] or in Chinese hamster ovary cells [36]. However, NAD^+ glycohydrolase-treated RL-PR-C hepatocyte cytosol, as well as pigeon erythrocyte cytosol, did stimulate both toxin catalyzed and endogenous ADP-ribosylation of the hepatocyte 55 000 dalton protein (Table III). The stimulation of ADP-ribosylation by erythrocyte cytosol (20-fold) was substantially greater than that caused by hepatocyte cytosol (3-fold). The effect of hepatocyte cytosol was, however, confined to hepatocyte ADP-ribosylation; it was without effect on erythrocyte ADP-ribosylation under conditions in which erythrocyte cytosol was very effective. Whether the stimulation of both endogenous and toxin-catalyzed ADP-ribosylation of hepatocytes by erythrocyte cytosol is due to the same cytosolic factor described by Enomoto and Gill [35] or to some other component of the cyto-

TABLE III

EFFECTS OF CYTOSOLS ON ADP-RIBOSYLATION

Hepatocyte and erythrocyte membranes and cytosol were prepared according to Gill and Meren [7]. Cholera toxin was activated immediately prior to use by incubation with 0.5% SDS/5 mM dithiothreitol for 10 min at 37°C. Membranes were incubated with or without 10 μg/ml cholera toxin in an incubation mixture containing nicotinamide [2,8-^{3}H]adenine dinucleotide (3500 cpm/pmol)/1 mM GTP/130 mM NaCl/0.1 mg/ml NaN_3/2 units/ml Trasylol in 10 mM Hepes buffer, pH 7.3; the ratio of cytosol to membrane protein was 3 : 1. The values shown are in terms of pmol ADP-ribose incorporated into the 42 000 dalton band (erythrocytes) or 55 000 dalton band (RL-PR-C hepatocytes), and represent the mean ± S.D. of duplicate determinations. n.d., not detectable. Less than 0.17 pmol (150 counts above background) cannot be accurately determined.

Additions	ADP-ribosylation (pmol/mg membrane protein)		
	Endogenous	Endogenous + cholera toxin	Cholera toxin (mean)
Pigeon erythrocyte membranes			
Control	n.d.	n.d.	
+ Erythrocyte cytosol	0.30 ± 0.03	0.74 ± 0.08	0.44
+ Hepatocyte cytosol	n.d.	n.d.	—
Rat hepatocyte membranes			
Control	0.52 ± 0.06	1.09 ± 0.11	0.57
+ Erythrocyte cytosol	9.77 ± 0.91	20.85 ± 1.34	11.08
+ Hepatocyte cytosol	1.66 ± 0.08	3.56 ± 0.42	1.90

sol, e.g., ADP-ribosyltransferase [31], must await purification of the factor. Since Enomoto and Gill's factor is thought to be between 10 000 and 20 000 daltons [37], it is tempting to speculate that the 55 000 dalton hepatocyte acceptor represents a permanent association of this factor with a 42 000 dalton membrane protein similar to the erythrocyte acceptor. It is also possible that both hepatocyte and erythrocyte cytosols contain factors that enhance endogenous and toxin-catalyzed ADP-ribosylation of hepatocytes and that the concentration of such factors is greater in erythrocyte cytosol. Such an explanation seems more likely than the former because hepatocyte cytosol was also capable of enhancing hepatocyte ADP-ribosylation, although to a lesser extent than erythrocyte cytosol, and could not substitute for erythrocyte cytosol in erythrocyte ADP-ribosylation. These observations would suggest that the requirements for hepatocyte ADP-ribosylation differ substantially from those for avian erythrocyte ADP-ribosylation.

Following completion of these studies, Hudson and Johnson [38] reported that the two cholera toxin substrates (M_r 42 000 and M_r 52 000—53 000) of S49 lymphoma cells [8] exhibited nearly identical peptide maps following limited proteolytic digestion. The relationship of the 55 000 dalton heptocyte acceptor to the 42 000 and 52 000—54 000 dalton acceptors of avian erythrocytes and S49 lymphoma cells, and the nature of cytosolic components are currently being explored.

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