Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 463-470

cAMP-DEPENDENT PHOSPHORYLATION OF RER PROTEINS FROM RAT LIVER: RELATIONSHIP WITH GTP-DEPENDENT MEMBRANE FUSION

Joël Lanoix and Jacques Paiement

Département d'anatomie, Faculté de Médecine
Université de Montréal, C.P. 6128, Succ. A
Montréal, Québec, CANADA
H3C 3J7

Received July 15, 1991

SUMMARY : Incubation of stripped rough microsomes (SRM) with the catalytic subunit of protein kinase A (PKA) permitted specific phosphorylation of seven proteins having relative molecular mass values of 55, 35, 23, 22.5, 22, 18.5 and 16.5 kDa (P55, P35 etc.). By two dimensional gel analysis, we compared these phosphoproteins with low-molecular-weight GTP-binding proteins and revealed that P23 and P22.5 co-migrated with known GTP-binding proteins. Next we examined the effect of cAMP-dependent phosphorylation on a GTP-dependent membrane function, membrane fusion. Quantitative analysis indicated no difference in the amount of membrane fusion obtained whether SRM were incubated in the absence or in the presence of PKA. Thus several rough microsomal proteins underwent cAMP-dependent phosphorylation and this post-translational modification did not affect GTP-dependent membrane fusion in a cell free system.

Low-molecular-weight GTP-binding proteins have been implicated in regulating intracellular transport at each step along the secretion pathway (1-7) as well as fusion of endocytic vesicles (8-10). Recently, $p34^{cdc2}$ protein kinase was observed to phosphorylate two low-molecular-weight GTP-binding proteins (11) and to inhibit fusion of endocytic vesicles (12). Thus phosphorylation of low-molecular-weight GTP-binding proteins by intracellular kinases could be a potent mechanism for modulating intracellular membrane interaction. Using RER membrane derivatives, in vitro membrane fusion was demonstrated to be a GTP-dependent process (13) indicating that GTP-binding proteins are probably involved in this phenomenon. Consistent with this suggestion was the identi-

The abbreviations used are : ATP : adenosine triphosphate; BSA : bovine serum albumin; cAMP : cyclic adenosine monophosphate; DTT : dithiothreitol; GTP : guanosine triphosphate; kDa : kilodalton; PKA : catalytic subunit of protein kinase A; pI : isoelectric point; RER : rough endoplasmic reticulum; SDS : sodium dodecyl sulfate; SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRM : stripped rough microsomes.

0006-291X/91 $1.50

fication of several GTP-binding proteins in subcellular fractions enriched in endoplasmic reticulum membranes (14-17). In this study we examined cAMP-dependent phosphorylation of rough microsomal proteins and compared the phosphoproteins with previously defined [α-^{32}P]GTP-binding proteins of rough microsomes. Then, we looked for a possible regulation by cAMP-dependent phosphorylation of GTP-dependent membrane fusion.

MATERIALS AND METHODS

Preparation of subcellular fractions : Rough microsomes were prepared from rat liver homogenates as previously described(18). The microsomes were stripped of associated ribosomes by using 5 mM pyrophosphate and washed with 3 mM-imidazole buffer, pH 7.4, containing 0.25 M sucrose(18). Protein concentrations were determined using the Lowry procedure(19) with bovine serum as standard.

In vitro phosphorylation of SRM proteins with PKA : cAMP-dependent phosphorylation of SRM proteins was done as described by Thiel et al. (20) with the exception that membranes were not preincubated 2 min at 95°C. Briefly 100 μg of SRM proteins were incubated in final volume of 100 μl containing 50 mM Hepes, pH 7.0, 10mM $MgCl_2$, 0.1 mM DTT, 10 μM [γ-^{32}P]ATP (25 Ci/mmol) and unless otherwise indicated 4 units/ml of PKA (Sigma Chemical Co. St-Louis, U.S.A., sp. act. 30-60 picomolar units/μg protein, one unit was defined as the amount of protein that phosphorylates one picomole of casein per minute at 30°C). Samples were incubated at 37°C during different times as indicated.

Single and double gel electrophoresis, electrophoretic blotting procedure and GTP binding to membrane proteins : Proteins were subjected to single dimensional gel electrophoresis (21) or double dimensional gel electrophoresis (17) in the presence of SDS on gradient polyacrylamide gels. The proteins were then transferred onto nitrocellulose sheets by the method of Towbin et al. (22). GTP binding to proteins on nitrocellulose sheets was carried out essentially as described by Bhullar and Haslam (23). Phosphorylation of proteins was quantitated by densitometric scanning of radioautograms of phosphorylated proteins. This was done using LKB UltroScan XL densitometer with GelScan XL software (LKB Pharmacia, Montréal, Canada).

Cell-free assay for membrane fusion: For cell-free membrane fusion assay, incubations of 200 μg of SRM proteins were carried out in 0.25 ml of the same medium as for cAMP-dependent phosphorylation (see above) plus 0.5 mM GTP. We determined that optimal conditions for membrane fusion and protein phosphorylation were the following; time of incubation of 15 min and PKA concentration of 16 units/ml. All incubations were done at 37°C. For morphology microsomes were fixed and processed for electron microscopy as previously described (24). Membrane fusion was quantitated by morphometry as previously described (25). Briefly electron micrographs of sectioned vesicles were prepared. The lengths of the membranes of 737 sectioned vesicles were measured and summated. Since small vesicles fuse to form large vesicles under fusion conditions the sum of the membrane lengths for 737 vesicles is always higher than that for an equivalent amount of vesicles incubated using non-fusion conditions. Therefore the total membrane length for vesicles incubated using non-fusion condition was subtracted from the total membrane length for vesicles incubated using fusion conditions and the difference expressed as a % of the amount of total membrane length for vesicles incubated using fusion conditions. This value gives the fusion index.

RESULTS

Stripped rough microsomes were incubated in the absence or in the presence of PKA and [γ-^{32}P]ATP. In the absence of PKA, radioautograms of single dimensional protein blots revealed only background labeling (Figures 1a & 1b). In the presence of PKA, seven proteins of relative molecular weight values of 55, 35, 23, 22.5, 22, 18.5 and 16 kDa (P55, P35 etc.) were specifically labeled (Figures 1c & 1d). Incubation of SRM proteins in the presence of GTP did not alter the phosphorylation profiles (compare Figures 1a with 1b and 1c with 1d). Because P23 and P22.5 showed similar electrophoretic mobilities to previously detected [α-^{32}P]GTP-binding proteins (17), we compared PKA phosphorylated proteins with [α-^{32}P]GTP-binding proteins by double dimensional gel electrophoresis. Radioautograms of two dimensional protein blots of SRM phosphoproteins revealed P23 as a constituent having pI values extending from 7.0 to 6.8 (Figure 2b). P22.5 was resolved as a constituent with pI values extending from 7.1 to 6.9 (Figure 2b). Comparison of these two phosphoproteins with [α-^{32}P]GTP-binding proteins revealed that P23 co-migrated with a 23 kDa [α-^{32}P]GTP-binding protein of similar pI values (Figure 2a). P22.5 co-migrated with [α-^{32}P]GTP-binding protein having similar pI values (Figure

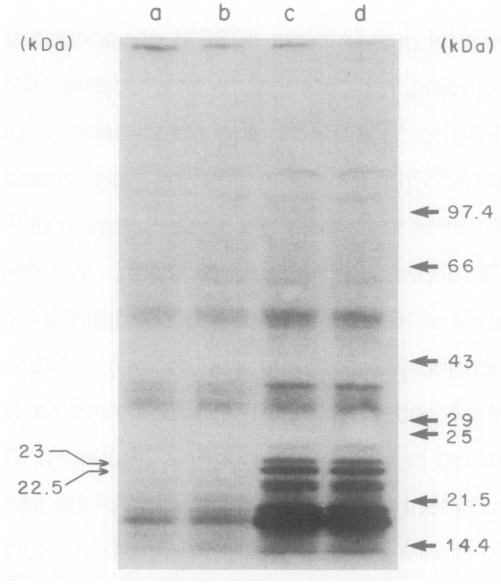

Fig. 1. cAMP-dependent phosphorylation of SRM proteins from rat liver. Phosphorylation of SRM proteins was done using 4 units/ml of PKA for 20 sec at 37°C as described in Materials and Methods. Proteins were subjected to SDS-PAGE, transferred onto nitrocellulose sheets followed by radioautography (radioautographic exposure 4.5 hr.) Lane a: SRM incubated without PKA and without GTP. Lane b: SRM incubated without PKA and with 0.5 mM GTP. Lane c: SRM incubated with PKA and without GTP. Lane d: SRM incubated with PKA and with 0.5 mM GTP. The level of migration of molecular weight markers is shown on the right.

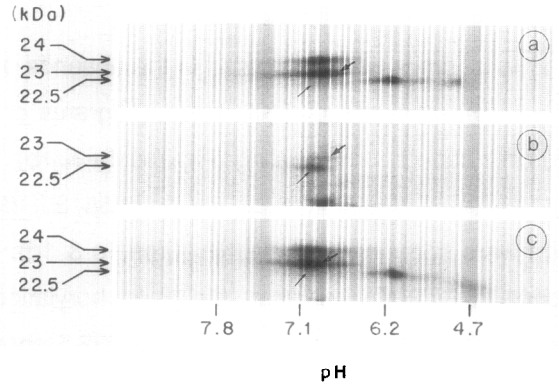

Fig.2. Comparison of phosphoproteins with [α-^{32}P]GTP-binding proteins in SRM after separation by two dimensional SDS-PAGE. SRM proteins were incubated with 16 units/ml of PKA for 15 min at 37°C as described in Materials and Methods. Proteins were then separated by two dimensional SDS-PAGE, transferred onto nitrocellulose sheets. In panel a, the microsomes were phosphorylated with PKA and cold ATP. The protein blot was then treated for detection of [α-^{32}P]GTP-binding proteins (radioautographic exposure, 15 hr). In panel b, the microsomes were phosphorylated with PKA and [γ-^{32}P]ATP. The protein blot was then exposed to revealed [γ-^{32}P]ATP phosphorylation (radioautographic exposure, 72 hr). In panel c, the same protein blot as in panel b was treated to reveal [α-^{32}P]GTP-binding and [γ-^{32}P]ATP phosphorylation (radioautographic exposure, 23 hr). Large arrows indicate the position of the 23 kDa phosphoprotein. Small arrows indicate the position of the 22.5 kDa phosphoprotein. Molecular weight values of corresponding labeled proteins are shown on left.

2a). When two dimensional protein blots of SRM phosphoproteins were subsequently probed for [α-^{32}P]GTP binding, radioautograms showed that P23 and P22.5 co-migrated with the 23 and 22.5 kDa [α-^{32}P]GTP-binding constituents (Figure 2c). These results suggested that several [α-^{32}P]GTP-binding proteins were phosphorylated by PKA. We therefore wanted to determine the effects of phosphorylation of SRM proteins particularly P23 and P22.5 on GTP-dependent membrane fusion. We determined that 15 min of incubation in the presence of 16 units/ml of PKA were optimal for detection of membrane fusion and protein phosphorylation (data not shown). After incubation using these conditions the amount of phosphorylation was observed to be as high as 11.5 fold compared to that observed in the absence of PKA (Table 1). Despite this difference in phosphorylation measurement of the amount of fusion of the same membranes revealed no significant difference (Table 2). We wondered whether saturation of phosphorylation sites was responsible for this negative result. To rule out this possibility, endogenous acceptors were dephosphorylated before cAMP-dependent phosphorylation using conditions where active unspecific endogenous phosphatases were observed to remove phosphate from phosphorylated proteins (data not shown). Subsequent phosphorylation revealed two new phosphoproteins one of 175 kDa, a second of 29 kDa and increased

TABLE 1

DENSITOMETRIC COMPARISON OF PHOSPHORYLATION OF STRIPPED ROUGH MICROSOMAL PROTEINS FROM RAT LIVER INCUBATED IN THE ABSENCE OR IN THE PRESENCE OF THE CATALYTIC SUBUNIT OF PROTEIN KINASE A

Phosphorylated proteins[b]	Absorbance[a] -PKA	Absorbance[a] +PKA	Fold increase
23	0.171 (0.054)	0.549 (0.557)	3.2 (10.3)
22.5	0.139 (0.135)	0.637 (1.558)	4.6 (11.5)

[a] Relative absorbance values of phosphorylated proteins incubated without or with PKA were determined as described in Materials and Methods. Values in parentheses represent results obtained from a separate experiment.

[b] Phosphorylation under optimal conditions of SRM, SDS PAGE, transfer onto nitrocellulose sheets and radioautography were done as described in Materials and Methods.

labeling of the 55 kDa phosphoproteins but no effect on the phosphorylation of proteins in the low-molecular-weight range (data not shown). This suggests that phosphorylation sites of low-molecular-weight GTP-binding proteins were probably not saturated prior to incubation.

TABLE 2

MORPHOMETRIC COMPARISON OF GTP-DEPENDENT FUSION OF STRIPPED ROUGH MICROSOMES FROM RAT LIVER INCUBATED IN THE ABSENCE OR IN THE PRESENCE OF THE CATALYTIC SUBUNIT OF PROTEIN KINASE A

Incubation condition	Mean membrane length of the vesicles μm	Fusion index %
- PKA	0.568 ± 0.308 (0.529 ± 0.466)	23.6 (17.9)
+PKA	0.517 ± 0.328 (0.570 ± 0.610)	16.0 (23.8)

Aliquots of the same membranes that were used in Table 1 were processed for electron microscopy and assayed for membrane fusion (see Materials and Methods). Values in parentheses represent results obtained from a separate experiment. Statistical analysis of mean membrane lengths of the vesicles incubated in the absence or presence of PKA revealed no difference between the means.

DISCUSSION

The incubation of SRM with PKA and [γ-^{32}P]ATP led to phosphorylation of seven proteins having relative molecular weights of 55, 35, 23, 22.5, 22, 18 and 16.5 kDa. P23 and P22.5 co-migrated with two [α-^{32}P]GTP-binding proteins and thus could be candidates involved in specific GTP-dependent membrane functions. We examined the relationship between PKA-dependent phosphorylation and GTP-dependent membrane fusion but were unable to observe modulation of fusion by phosphorylation (Tables 1 and 2). We conclude that cAMP-dependent phosphorylation probably does not regulate GTP-dependent membrane fusion. The low-molecular-weight phosphoproteins identified in our study could modulate other known GTP-dependent functions in RER membranes such as core glycosylation (26), CDP-diacylglycerol formation (27), membrane permeability changes (28), calcium release (29) and integration of nascent proteins into RER (30).

We do not believe the low-molecular-weight phosphoproteins we have observed correspond to H-ras, K-ras, rap 1A or rap 1B even though these low-molecular-weight GTP-binding proteins have been demonstrated to be substrates for cAMP-dependent protein kinase (31-33). Indeed, anti-p21 ras antibodies were unable to label rough microsomes by immunocytochemistry and were unable to immunoprecipitate ras protein from these same membranes (34) and antibody M-90 which recognized rap proteins (35, 36) failed to detect any phosphorylated proteins by immunoblot detection (Lanoix, J., Paiement, J. and Lacal, J.C., unpublished observations).

The low-molecular-weight phosphoproteins we describe could be relate to the cAMP-dependent phosphorylated microsomal proteins detected in rat parotid gland (37, 38). Phosphoproteins P55 and P35 may correspond to hepatic cytochrome p-450 and ribosomal protein S6 respectively since cAMP-dependent phosphorylation of these proteins has previously been demonstrated (39, 40). Recent findings suggest that three enzymes involved in triglyceride synthesis were sensitive to cAMP-dependent phosphorylation (41) raising the possibility that one or several phosphoproteins we define could be related to these enzymes.

In summary, cAMP-dependent phosphorylation was demonstrated to proteins several of which are probably GTP-binding proteins in rat liver rough microsomes. This post-translational modification does not influence GTP-dependent membrane fusion but may be more relevant for other RER functions.

ACKNOWLEDGMENTS

We thank Dr. J-C. Lacal for the gift of monoclonal antibody M-90, Line Roy for assistance with the morphometric studies, Jean Léveillé for photographic work and

Emilienne Lambert for art work. The work was supported by grants from the Medical Research Council of Canada, the Fonds de la Recherche en Santé du Québec, the Cancer Research Society Inc. (MT-7325, 850039 and MD37960 to JP), and by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (studentship, J.L).

REFERENCES

1. Bacon, R.A., Salminen, A., Ruohola, H., Novick, P., and Ferro-Novick, S. (1989) J. Cell. Biol. 109, 1015-1022.
2. Beckers, C.J.M., and Balch, W.E. (1989) J. Cell. Biol. 108, 1245-1256.
3. Melançon, P., Glick, B.S., Malhotra, V., Weidman, P.J., Serafini, T., Gleason,M.L., Orci, L., and Rothman, J.E. (1987) Cell 51, 1053-1062.
4. Segev, N., Mulholland, J., and Bothstein, D. (1988) Cell 52, 915-924.
5. Barrowman, M.M., Cockcroft, S., and Gomperts, B.D. (1986) Nature 319, 504-507.
6. Salminen, A., and Novick, P.J. (1987) Cell 49, 527-538.
7. Padfield, P.J., Ding, T-G., and Jamieson, J.D. (1991) Biochem. Biophys. Res. Comm. 174, 536-541.
8. Mayorga, L.S., Diaz, R., and Stahl, P.D. (1989) Science 244, 1475-1477.
9. Wessling-Resnick, M., and Braell, W.A. (1990) J. Biol. Chem. 265, 16751-16759.
10. Gorvel, J.P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) Cell 64, 915-925.
11. Bailly, E., McCaffrey, M., Touchot, N., Zahraoui, A., Goud, B., and Bornens, M. (1991) Nature 350, 715-718.
12. Tuomikoski, T., Felix, M.A., Dorée, M., and Gruenberg, J. (1989) Nature 342, 942-945.
13. Paiement, J., Beaufay, H., and Godelaine, D. (1980) J. Cell.Biol. 86, 29-37.
14. Robinson, A., and Austen, B. (1987) Febs. Lett. 218, 63-67.
15. Audigier, Y., Nigam, S.K., and Blobel, G. (1988) J. Biol. Chem. 263, 16352-16357.
16. Nigam, S.K. (1990) Proc. Natl. Acad. USA 87, 1296-1299.
17. Lanoix, J., Roy, L., and Paiement, J. (1989) Biochem. J. 262, 497-503.
18. Paiement, J., and Bergeron, J.J.M. (1983) J. Cell. Biol. 96, 1791-1796.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall. (1951) J. Biol. Chem. 193, 265-275.
20. Thiel, G., Schmidt, W.E., Meyer, H.E., and Söling, H-D. (1988) Eur. J. Biochem. 170, 643-651.
21. Paiement, J. (1984) Exp. Cell. Res. 151, 354-366.
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. USA 76, 4350-4354.
23. Bhullar, R.P., and Haslam, R.J. (1987) Biochem. J. 245, 617-620.
24. Paiement, J., Rindress, D., Smith, C.E., Poliquin, L., and Bergeron, J.J.M. (1987) Biochem. Biophys. Acta 898, 6-22.
25. Paiement, J., Dominguez, J.M., Guénette, A., and Roy, L. (1991) Biochem. Biophys. Res. Comm. 176, 1494-1500.
26. Godelaine, D., Beaufay, H., and Wibo., M. (1977) Proc. Natl. Acad. Sci. USA 74, 1095-1099.
27. Sribney. M., Dove, J.L., and Lyman, E.M. (1977) Biochem. Biophys. Res. Commun. 79, 749-755.
28. Nicchitta, C.V., Joseph, S.K., and Williamson, J.R. (1987) Biochem. J. 248, 741-747.
29. Dawson, A.P. (1985) FEBS. Lett. 185, 147-150.
30. Wilson. C., Connolly, T., Morrison, T., and Gilmore R. (1988) J. Cell. Biol. 107, 69-77.
31. Saikumar, P., Ulsh, L.S., Clanton, D.J., Huang, K-P., and Shih, T.Y. (1988) Oncogene Res. 3, 213-222.

32. Lerosey, I., Pizon, V., Tavitian, A., and de Gunzburg, J. (1991) Biochem. Biophys. Res. Comm. 175, 430-436.
33. Fischer, T.H., and White II, G.C. (1987) Biochem. Biophys. Res. Comm. 149, 700-706.
34. Dominguez, J.M., Lanoix, J., and Paiement, J. (1991) Exp. Cell. Res. 192, 137-147.
35. Winegar, D.A., Ohmstede, C-A., Chu, L., Reep, B., and Lapetina, E.G. (1991) J. Biol. Chem. 266, 4375-4380.
36. Fischer, T.H., Gatling, M.N., Lacal, J-C., and White II, G.C. (1990) J. Biol. Chem. 265, 19405-19408.
37. Spearman, T.N., Hurley, K.P., Olivas, R., Ulrich, R.C., and Butcher, F.R. (1984) J. Cell. Biol. 99, 1354-1363.
38. Jahn, R., Unger, C., and Söling, H-D. (1980) Eur. J. Biochem. 112, 345-352.
39. Pyerin, W., and Taniguchi, H. (1989) Embo J. 8, 3003-3010.
40. Jahn, R., and Söling, H-D. (1983) Febs Lett. 153, 71-76.
41. Söling, H-D., Fest, W., Schmidt, T., Esselmann, H., and Bachmann, V. (1989) J. Biol. Chem. 264, 10643-10648.