Activation of Glycogen Phosphorylase with 5-Aminoimidazole-4-Carboxamide Riboside (AICAR)

ASSessment of GlycoGen as a Precursor of Mannosyl Residues in GlycoConjugates*

Received for publication, January 14, 2004
Published, JBC Papers in Press, January 16, 2004, DOI 10.1074/jbc.M400431200

Jie Shang and Mark A. Lehrman‡
From the Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041

The experimental evaluation of the contribution of glycogen phosphorylase (GP) to biochemical pathways is limited to methods that raise cAMP, activating the cAMP-dependent protein kinase/phosphorylase kinase/GP cascade. Such methods convert the unphosphorylated form, “GPb,” which catalyzes glycogenolysis only in the presence of appropriate allosteric activators such as AMP, to the phosphorylated, constitutively activated form, “GPa.” However, activation of GP in this way is indirect; requires a functional cAMP kinase cascade, and is complicated by other actions of cAMP. Here, we demonstrate a strategy for the experimental manipulation of GP in intact dermal fibroblasts, involving activation by the membrane-permeable adenosine analog 5-aminoimidazole-4-carboxamide riboside (AICAR) and inhibition by caffeine and Pfizer compound CP-91149, which bind to GP at distinct sites. Potential complications because of activation of AMP-activated protein kinase by AICAR were assessed with metformin, which activates this kinase but does not activate GP. Using this strategy, we show that glycogen can be a significant and regulatable precursor of mannosyl units in lipid-linked oligosaccharides and glycoproteins.

Glycogen, a glucose polymer, is a key store of hexose in mammalian cells. Its formation from UDP-Glc and breakdown to Glc-1-P is catalyzed, respectively, by glycogen synthase and glycogen phosphorylase (GP). GP exists in two forms, depending upon its phosphorylation state (1). “GPb,” the unphosphorylated form, can be stimulated by allosteric activators such as AMP. Alternatively, phosphorylase kinase can convert the enzyme into its phosphorylated, covalently activated form “GPa,” which no longer requires allosteric activation. The regulation of GP may be complicated further by phosphatases and phosphatase inhibitors and the abilities of enzymes such as AMP-activated protein kinase (AMPK) (2) and protein phosphatase-1 (3) to bind to glycogen.

Recently, interest has been generated in glycogen as a source of hexose units in N-linked glycoproteins (4, 5). Protein N-linked glycosylation requires the lipid-linked oligosaccharide (LLO) Glc3Man9GlcNAc2-P-P-dolichol. The oligosaccharide unit is transferred by oligosaccharyl transferase to asparaginyl residues on nascent proteins (6). LLO synthesis requires contributions of enzymes in both the cytoplasm and the endoplasmic reticulum. In the cytoplasm, precursor nucleotide sugars are synthesized. In contrast, the endoplasmic reticulum is the site of assembly of the LLO, requiring UDP-GlcNAc, GDP-mannose, and UDP-glucose as well as the lipids mannose-P-dolichol and glucose-P-dolichol, which are synthesized from GDP-mannose and UDP-glucose, respectively.

Two independent approaches have suggested that phosphorylisis of glycogen to form Glc-1-P can be stimulated under particular forms of cellular stress and that the Glc-1-P can be converted sequentially to Glc-6-P, Fru-6-P, Man-6-P, Man-1-P, and GDP-mannose in sufficient quantities to substantially enhance glycoconjugate synthesis. Impaired LLO mannosylation in cultured 3T3-L1 and Chinese hamster ovary-KI cells correlated with glycogen depletion resulting from glucose starvation (4). In addition, increased glucose phosphate production and glycolysis were coincident with endoplasmic reticulum stress and improved LLO mannosylation (5). Although such experiments suggest that glycogen may be a significant precursor of mannosyl units for glycoconjugate synthesis, in each case glycogenolysis was activated indirectly. This problem could be solved by deliberate activation of GP. However, current approaches for experimental stimulation of GP in intact cells require activating the cAMP-dependent protein kinase/phosphorylase kinase cascade by adding an appropriate receptor agonist, an activator of adenyl cyclase, or a membrane-permeable form of cAMP. Activation of phosphorylase kinase by these methods results in the conversion of GPs to GPs, but other pathways also respond to cAMP, so it would be difficult to attribute any effects on LLO synthesis solely to activation of GP.

Here, we examined 5-aminoimidazole-4-carboxamide riboside (AICAR), which like metformin (7) is widely reported to cause activation of AMPK in cells (8). AICAR enhanced LLO extension as anticipated, but surprisingly its mechanism was inconsistent with that of metformin (9). Through a pharmacological approach, we show that AICAR enhanced LLO synthesis primarily by stimulating GP not AMPK.

EXPERIMENTAL PROCEDURES

Reagents—Compound CP-91149 (10) was a generous gift of Pfizer. AICAR (number A61700) was from Toronto Research Chemicals. AICAR-P (ZMP, number A1393), metformin (number D5035), and caffeine (number C0750) were from Sigma. All of the above reagents were prepared as stock solutions in water. Cell culture media were from Invitrogen, and sera were from Atlanta Biologicals. [2-3H]Mannose (10–20 Ci/mmol) was from Amersham Biosciences, and...
Because metformin is a positive modulator of LLO synthesis (9) we examined AICAR, as it has been widely reported that treatment of cells with AICAR, like metformin, activates AMPK (8). This requires intracellular conversion of AICAR to the phosphorylated nucleotide 6-aminoimidazole-4-carboxamide ribotide (designated AICAR-P for simplicity) by adenosine kinase (15, 16). AICAR-P allosterically activates purified AMPK, although with a substantially lower affinity than AMP (17, 18). Depending upon the cell type and the experimental system, AICAR-P in intact cells appears to act primarily either by allosteric activation of AMPK (19) or by making AMPK a better substrate for the upstream-activating kinase complex containing LKB1 (20). In any case, the mechanism of AICAR-P differs from that of metformin, which does not activate the isolated enzyme but can activate AMPK in intact cells (7, 21).

As shown in Fig. 1, AICAR enhanced extension of LLO intermediates (a and b) and increased the proportion of Glc3Man5GlcNAc2-P-P-dolichol for treatment with AICAR-P in intact cells and 29% in AICAR-treated cells), which stimulates LLO synthesis mainly by enhancing mannose uptake (9). However, the effect of metformin on mannose transport (1.8-fold (9)) was greater than that of AICAR (1.3 ± 0.02-fold (n = 22); Fig. 2b), suggesting different mechanisms of action. Like metformin (9), AICAR did not increase transport of 2-deoxyglucose in fibroblasts (Fig. 2a).

To verify that AICAR was less dependent than metformin upon mannose uptake for its effect on LLO synthesis, incubations were done with 40 μCi/ml [3H]mannose, 40 μCi/ml [3H]mannose with AICAR, or 60 μCi/ml [3H]mannose. As shown in Fig. 2c, compared with 40 μCi/ml [3H]mannose alone, treatment of cells with AICAR has less of an effect on mannose uptake than using 60 μCi/ml [3H]mannose. However, the relative amounts of Glc3Man5GlcNAc2-P-P-dolichol were 1.6% for 40 μCi/ml [3H]mannose alone, 31.7% for 40 μCi/ml [3H]mannose with AICAR, and 3.9% for 60 μCi/ml [3H]mannose (Fig. 2, d–f), indicating that mannose uptake was not a significant factor for AICAR treatment of fibroblasts.

**AICAR-P Activates Glycogen Phosphorylase in Fibroblast Extracts**—AICAR/AICAR-P has been reported to activate the AMP-dependent form of glycogen phosphorylase, GPb, in skeletal (22) and cardiac (19) muscle systems. In both cases AICAR-P activated GP in muscle homogenates, but distinct mechanisms were suggested. Noting that phosphorylase kinase had been reported to be a possible substrate of AMPK (23) (although a more recent study failed to reach a similar conclusion (24)), in skeletal muscle GPb activation was proposed to involve the
binding of AICAR-P to AMPK with a sequential phosphorylation of phosphorylase kinase and phosphorylation of GPa (22) rather than to involve the binding of AICAR-P directly to the AMP activation site on GPa. However, the homogenate experiments did not appear to include ATP or an ATP-generating system and were done under conditions in which all three enzymes in the cascade were diluted 130-fold with respect to the homogenate. In contrast, in cardiac muscle, activation by the effects of AICAR was proposed to involve direct allosteric activation of GP by AICAR-P rather than activation of a kinase cascade. This was supported by the observations that treatment of muscle with AICAR led to increased glycogenolysis, decreased glycogen content, no change in the phosphorylation state of AMPK, and no evidence of covalent activation of AMPK or GP. However, phosphorylation of a substrate of AMPK suggested that AICAR did activate AMPK allosterically.

We found that a commercial preparation of muscle GPa (Sigma) could be activated by AICAR-P as well as AMP and in the absence of AMP (data not shown), which is highly consistent with direct allosteric activation of GPa by AICAR-P. Importantly, 5 mM AMP and 5 mM AICAR-P, alone and in combination, stimulated GP activity in fibroblast extracts (Fig. 3, upper panel, lanes 1, 4, 7, and 10), whereas AICAR and metformin did not (data not shown). In these experiments cytoplasmic components were highly diluted, and reactions were done in the absence of ATP. Therefore, the results are consistent with direct allosteric activation and in discord with a sequential enzyme cascade mechanism.

AICAR-dependent LLO Extension Is Blocked by Inhibitors of Glycogen Phosphorylase—To determine whether AICAR enhanced LLO extension by activating GP in intact fibroblasts, two inhibitors of GP were evaluated. Caffeine, which binds to the nucleoside-inhibitory site of GP (1), had no effect on GP activity by itself (data not shown). However, it blocked the stimulation of GP in fibroblast extracts by AMP and AICAR-P (Fig. 3, upper panel, lanes 3, 6, 9, and 12). Using the system for LLO synthesis introduced in Fig. 1, caffeine by itself did not stimulate mannosylation of LLOs (Fig. 3, lower panel, a, d, f, and j). However, as shown in Fig. 3, b and g, caffeine eliminated the stimulatory effect of AICAR on LLO extension (the chromatograms shown are representative of four independent experiments). Caffeine did not inhibit the effect of 0.1 mM mannose on LLO synthesis (Fig. 3, c and h), showing that it did not inhibit the relevant mannosyltransferases nonspecifically. Although both GP and AMPK are potential targets for AICAR-P, metformin is known only to activate the latter. GP in fibroblast extracts was not activated by metformin (data not shown), and the ability of metformin to stimulate LLO extension was not blocked by caffeine (Fig. 3, e and j).

Pfizer compound CP-91149 is a highly specific inhibitor of GP, acting both in vitro and in vivo (10). CP-91149 binds to a novel site on GP (25) distinct from the caffeine binding site. Therefore, CP-91149 was used to verify whether AICAR acted by stimulating GP. Although CP-91149 was identified as an inhibitor of hepatic GPs, it also inhibited commercial preparations of muscle GPs in the absence of AMP and, somewhat unexpectedly, muscle GPa activated by AMP (data not shown). As shown in Fig. 3 (upper panel, lanes 2, 5, 8, and 11), CP-91149 inhibited both AMP- and AICAR-P-activated GP in fibroblasts extracts.

CP-91149 was then tested as an inhibitor of the AICAR-stimulated extension of LLOs in intact cells (Fig. 4, upper panel). Like caffeine, 20 μg/ml CP-91149 by itself did not promote LLO extension (Fig. 4, a and c), but it blocked the stimulatory effect of AICAR (b and d). This confirmed that AICAR acted by stimulating GP. The inhibitory effects of CP-91149 were dose-dependent, with less inhibition by 10 μg/ml. It was noticed that the efficacy of the AICAR treatment for LLO synthesis was variable, and the 10 μg/ml dose of CP-91149 was considerably more effective as an inhibitor when the efficacy of AICAR was lower (Fig. 4, lower panel). When AICAR increased Glc₃Man₂GlcNAc₂-P-dolichol above 20% of the LLO pool, 10 μg/ml CP-91149 had no discernable effect. Consistent with previous results, the effects of metformin on LLO extension were not blocked by CP-91149 (not shown).

DISCUSSION

AICAR differed from metformin in three important ways, in support of the conclusion that the primarily targets of AICAR and metformin in fibroblasts are GP and AMPK,
AICAR-P (2 was included in assays but not to AICAR. It is interesting that HeLa cells lack by an atypical AMPK cascade that is responsive to metformin sibility is that mannose transport in fibroblasts is controlled detect and have a much greater significance. Another pos- ing both enzymes, but GP activation may be more readily a greater ratio of GP to AMPK. Thus, AICAR may be activat- Perhaps, compared with other systems, these cells may have systems, and the fibroblasts were responsive to metformin. AICAR has been reported to activate AMPK in a number of fibroblast extracts. These results were unexpected, because take. (iii) AICAR-P, but not metformin, stimulated GPb in highl dependent upon its ability to stimulate mannose up- (ii) The effect of AICAR but not metformin on LLO extension was blocked by caffeine and CP-91149. (i) The effect of AICAR but not metformin on LLO extension was arbitrarily assigned a value of 1. Assays were performed in the absence (lanes 1–3) or presence of 5 mM concentrations of the activators AICAR-P (lanes 4–6, 10–12) or AMP (lanes 7–12). 10 µM CP-91149 was included in assays 2, 5, 8, and 11, and 5 mM caffeine was included in assays 3, 6, 9, and 12. Lower panel, LLOs were analyzed after fibroblasts were grown as in Fig. 1 without mannosylation enhancers (a, c, d, f, h, and i), with 2 mM AICAR for 7 h (b, g), or with 2 mM metformin for 24 h (c, j). Incubations had no caffeine (a–e) or 5 mM caffeine for 7 h (f–h) or 24 h (i and j). Unlabeled 0.1 mM mannose was added during the [1H]mannose labeling step in c and h. respectively. (i) The effect of AICAR but not metformin on LLO extension was blocked by caffeine and CP-91149. (ii) The effect of metformin but not AICAR on LLO extension was highly dependent upon its ability to stimulate mannose uptake. (iii) AICAR-P, but not metformin, stimulated GPb in fibroblast extracts. These results were unexpected, because AICAR has been reported to activate AMPK in a number of systems, and the fibroblasts were responsive to metformin. Perhaps, compared with other systems, these cells may have a greater ratio of GP to AMPK. Thus, AICAR may be activating both enzymes, but GP activation may be more readily detected and have a much greater significance. Another possibility is that mannose transport in fibroblasts is controlled by an atypical AMPK cascade that is responsive to metformin but not to AICAR. It is interesting that HeLa cells lack functional LKB1 (20), but mannose transport in HeLa cells is responsive to metformin (9). There is little doubt that AICAR was converted to AICAR-P in fibroblasts, as it is in many mammalian systems (8), especially because AICAR did not activate GP in extracts. Estimates based upon formation of AICAR-P from AICAR in Chi- nese hamster ovary cells (16) and muscle (15) are consistent with intracellular concentrations in the millimolar range suf- ficient to activate GP. A canine plasma AICAR concentration of 0.34 mM resulted in approximate AICAR-P tissue concentra- tions of 0.14 mM in skeletal muscle and 0.81 mM in cardiac muscle (15); extrapolation to 2 mM AICAR (as used in this study) therefore suggests fibroblast intracellular AICAR-P concentra- tions of ~1–5 mM. Similarly, Chinese hamster ovary-K1 cells treated for 7 h with 0.7 mM AICAR accumulated 11 nmol of AICAR-P/mg of cellular protein (16). In our hands, packed fibroblasts giving a volume of 1 ml would contain roughly 0.5 g of total protein, suggesting that incubation of fibroblasts with 2 mM AICAR for 7 h (as done here) might result in ~15 mM intracellular AICAR-P. In any case, these results show that the combination of an activator (AICAR/AICAR-P) with inhibitors that act by distinct mechanisms (caffeine and CP-91149) can be used for the exper- imental evaluation of GP. This strategy should be applicable
whether AICAR-P activates GP allosterically or stimulates AMPK to phosphorylate phosphorylase kinase, because the two inhibitors are effective for GPb and GPa. These results also demonstrate that the Glc-1-P resulting from glycogenolysis can be a significant source of hexose units for glycoconjugate assembly and provide a proof-of-principle for activation of glycogenolysis by the unfolded protein response as the cause of enhanced LLO extension (11, 12). Thus, the GP modulators used here provide glycobiologists with a novel approach for experimentally altering glycoconjugate synthesis. GP and AMPK respond to variations in energy metabolism, which can be caused by transient drops in cytoplasmic hexose concentrations. Such fluctuations are likely to affect LLO synthesis and energy metabolism in tandem. Therefore, GP and AMPK may have important roles in maintaining a consistent supply of hexose for LLOs and presumably for other glycoconjugates, such as glycosylphosphatidylinositol anchors, that require GDP-mannose.

Acknowledgments—We thank Charisse Estess for performing preliminary glycogen phosphorylase assays and Biswanath Pramanik for assistance with cell culture. We are grateful for the gift of CP-91149 and preliminary glycogen phosphorylase assays and Biswanath Pramanik for assistance with cell culture. We are grateful for the gift of CP-91149 and the tol anchors, that require GDP-mannose.

REFERENCES

1. Johnson, L. N. (1992) FASEB J. 6, 2274–2282
2. Polekhina, G., Gupta, A., Michell, B. J., van Dendersen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) Curr. Biol. 13, 867–871
3. Newgard, C. B., Brady, M. J., O'Doherty, R. M., and Saltiel, A. R. (2000) Diabetes 49, 1967–1977
4. McMahon, R. J., and Frost, S. C. (1996) Am. J. Physiol. 270, E640–E645
5. Gill, A., Gao, N., and Lehrman, M. A. (2002) J. Biol. Chem. 277, 44747–44753
6. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
7. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fuji, N., Musi, N., Hirschman, M., Goodyear, L. J., and Moller, D. E. (2001) J. Clin. Invest. 108, 1167–1174
8. Winder, W. W., and Hardie, D. G. (1999) Am. J. Physiol. 277, E1–E10
9. Shang, J., and Lehrman, M. A. (2004) J. Biol. Chem. 279, 9703–9712
10. Martin, W. H., Hoover, D. J., Armento, S. J., Stock, I. A., McPherson, R. K., Danley, D. E., Stevenson, R. W., Barrett, E. J., and Treadway, J. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1176–1181
11. Doerrler, W. T., and Lehrman, M. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13050–13055
12. Shang, J., Korner, C., Freeze, H., and Lehrman, M. A. (2002) Glycoconjugate J. 19, 307–317
13. Zeng, Y., and Lehrman, M. A. (1991) Anal. Biochem. 193, 266–271
14. Anand, M., Rush, J. S., Ray, S., Doucey, M. A., Weik, J., Ware, F. E., Hofsteenge, J., Waechter, C. J., and Lehrman, M. A. (2001) Mol. Biol. Cell 12, 487–501
15. Sabina, R. L., Kerrutine, K. H., Boyd, R. L., Holmes, E. W., and Swain, J. L. (1982) J. Biol. Chem. 257, 10178–10183
16. Sabina, R. L., Patterson, D., and Holmes, E. W. (1985) J. Biol. Chem. 260, 6107–6114
17. Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995) Eur. J. Biochem. 239, 558–565
18. Henin, N., Vincent, M. F., and Van den Bergh, G. (1996) Biochim. Biophys. Acta 1290, 197–203
19. Langner, S. L., Wambolt, R. B., Parsons, H. L., Brownsey, R. W., and Allard, M. F. (2000) Am. J. Physiol. 278, R936–R944
20. Hawley, S. A., Boudreau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessio, D. R., and Hardie, D. G. (2003) J. Biol. Chem. 278, 28–28.15
21. Hawley, S. A., Gadalla, A. E., Olsen, G. S., and Hardie, D. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13050–13055
22. Young, M. E., Radda, G. K., and Leighton, B. (1996) FEBS Lett. 382, 43–47
23. Carling, D., and Hardie, D. G. (1989) Biochim. Biophys. Acta 1012, 81–86
24. Beyer, A., Kitzerow, A., Crute, B., Kemp, B. E., Witters, L. A., and Heilmeyer, L. M. G., Jr. (2000) J. Clin. Investig. 108, 1167–1174
25. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
26. Gill, A., Gao, N., and Lehrman, M. A. (2002) J. Biol. Chem. 277, 44747–44753
27. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
28. Martin, W. H., Hoover, D. J., Armento, S. J., Stock, I. A., McPherson, R. K., Danley, D. E., Stevenson, R. W., Barrett, E. J., and Treadway, J. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1176–1181
29. Danley, D. E., Stevenson, R. W., Barrett, E. J., and Treadway, J. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1176–1181
30. Shang, J., Korner, C., Freeze, H., and Lehrman, M. A. (2002) Glycoconjugate J. 19, 307–317
31. Zeng, Y., and Lehrman, M. A. (1991) Anal. Biochem. 193, 266–271
32. Anand, M., Rush, J. S., Ray, S., Doucey, M. A., Weik, J., Ware, F. E., Hofsteenge, J., Waechter, C. J., and Lehrman, M. A. (2001) Mol. Biol. Cell 12, 487–501
33. Sabina, R. L., Kerrutine, K. H., Boyd, R. L., Holmes, E. W., and Swain, J. L. (1982) J. Biol. Chem. 257, 10178–10183
34. Sabina, R. L., Patterson, D., and Holmes, E. W. (1985) J. Biol. Chem. 260, 6107–6114
35. Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995) Eur. J. Biochem. 239, 558–565
36. Henin, N., Vincent, M. F., and Van den Bergh, G. (1996) Biochim. Biophys. Acta 1290, 197–203
37. Langner, S. L., Wambolt, R. B., Parsons, H. L., Brownsey, R. W., and Allard, M. F. (2000) Am. J. Physiol. 278, R936–R944
38. Hawley, S. A., Boudreau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessio, D. R., and Hardie, D. G. (2003) J. Biol. Chem. 278, 28–28.15
39. Hawley, S. A., Gadalla, A. E., Olsen, G. S., and Hardie, D. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13050–13055
40. Young, M. E., Radda, G. K., and Leighton, B. (1996) FEBS Lett. 382, 43–47
41. Carling, D., and Hardie, D. G. (1989) Biochim. Biophys. Acta 1012, 81–86
42. Beyer, A., Kitzerow, A., Crute, B., Kemp, B. E., Witters, L. A., and Heilmeyer, L. M. G., Jr. (2000) J. Clin. Investig. 108, 1167–1174
43. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664