BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 179–183 (1997)
ARTICLE NO. RC965927

Antipeptide Antibodies as Probes of Subunit-Dependent Structural Changes in the Regulatory Domain of the Gamma-Subunit of Phosphorylase Kinase

Wendy P. Wangsgard,* Maitrayee Dasgupta,†,[1] and Donald K. Blumenthal‡,[2]
*Department of Pharmacology & Toxicology, University of Utah, Salt Lake City, Utah 84112;
†Department of Biochemistry, The University of Texas Health Center at Tyler, Tyler, Texas 75710;
and ‡Department of Biochemistry, University of Utah, Salt Lake City, Utah 84112

Received November 27, 1996

The γ-subunit of phosphorylase kinase contains a protein kinase catalytic domain (residues 20–276) and a regulatory domain (residues 276–386). The purpose of the present investigation was to develop monospecific antibodies against four synthetic γ-subunit regulatory domain peptides (PhK1: 362–386; PhK5: 342–366; PhK9: 322–346; PhK13: 302–326) to use as probes to study the structure of the regulatory domain. Each affinity-purified antibody was characterized with regard to its ability to bind three different structural forms of the γ-subunit: the isolated γ-subunit, the γ-δ complex, and the holoenzyme complex $(\alpha\beta\delta\gamma)_4$. Of the four antibodies, binding of affinity-purified anti-PhK13 was most affected by alterations in γ-subunit interactions. Taken together, the data from this investigation indicate that the regulatory domain of the γ-subunit can assume different immunochemically distinguishable conformations as the result of interactions among the α-, β-, γ-, and δ-subunits of phosphorylase kinase. © 1997 Academic Press

The skeletal muscle form of phosphorylase kinase is a multimeric holoenzyme complex with the subunit composition $(\alpha\beta\delta\gamma)_4$ and a molecular weight of 1.3×10^6 (1-3). Molecular weights of the individual subunits are α = 145,000, β = 128,000, γ = 44,673, and δ = 16,680 (reviewed in (4)). The α- and β-subunits are regulatory subunits that are phosphorylated by the cAMP-dependent protein kinase and by autophosphorylation (4). The γ-subunit is the catalytic subunit (5-9), and the δ-subunit is identical to calmodulin (1). Phosphorylase kinase is different from most other calmodulin-regulated enzymes in that the δ-subunit stays tightly associated in the holoenzyme in the absence of calcium. The details of subunit interactions in the phosphorylase kinase holoenzyme complex are poorly understood, mainly because of its large size and complex oligomeric structure. Evidence from subunit dissociation studies have indicated a strong association between the γ-subunit and δ-subunits (6), whereas cross-linking studies have indicated interactions between the γ-subunit and the α-, β-, and δ-subunits (10, 11). Thus, the catalytic γ-subunit appears to interact directly with each of the other subunits in the holoenzyme, although the sites of interaction on each subunit are still poorly defined.

Sequence analysis of the δ-subunit shows a lack of homology between the C-terminal 110 residues of the δ-subunit and other protein kinases (8). Based on this lack of sequence similarity, Reimann and coworkers suggested that this region of the γ-subunit might be involved in oligomeric interactions (8). Subsequent experiments with proteolytic (12) and genetically-engineered fragments (13, 14) of the γ-subunit have demonstrated the importance of the C-terminal 90-100 residues in δ-subunit interactions. Synthetic peptide studies (15) have defined the δ-subunit interaction domain as consisting of two noncontiguous subdomains, $\gamma_{342\text{-}366}$ (termed PhK5) and $\gamma_{302\text{-}326}$ (termed PhK13). The sites of interaction between the γ-subunit and the α- and β-subunits have not yet been identified.

The purpose of the present investigation was to develop a set of antipeptide antibodies specific for defined regions within the γ-subunit regulatory domain and to use these monospecific antibodies to detect conformational changes in the γ-subunit regulatory domain due to subunit interactions. The results of these and related studies (16) indicate that the conformation of the γ-

[1] Present address: Department of Biochemistry, Ballygung Science College, Calcutta University, 35, Ballygung Circular Road, Calcutta, India.
[2] Corresponding author: Department of Pharmacology & Toxicology, 112 Skaggs Hall, University of Utah, Salt Lake City, UT 84112. Fax: (801) 585-5111. E-mail: dkb@max.pharm.utah.edu.

0006-291X/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

subunit regulatory domain is altered by γ-subunit interactions with the α-, β- and δ-subunits, suggesting that the regulatory α-, β- and δ-subunits can exert at least some of their effects on the catalytic activity of phosphorylase kinase by altering the conformation of the γ-subunit regulatory domain.

MATERIALS AND METHODS

Protein preparation. Phosphorylase kinase was purified from rabbit skeletal muscle by the procedure of Cohen (2). Calmodulin was purified from bovine testes as previously described (15). The γ-subunit of phosphorylase kinase was isolated from the phosphorylase kinase holoenzyme complex using reversed-phase HPLC as described by Crabb and Heilmeyer (17), except that a Vydac C-4 (5 mm, 0.46 $\times$ 25 cm) analytical column was used instead of a C-18 column. Formation of the γ-δ complex was performed using the procedure described by Kee and Graves (7). The reactivation reaction mixture contained 50 mM Tris, 50 mM β-glycerophosphate, pH 8.0, 2 mM dithiothreitol, 0.1 mM $CaCl_2$, 3 μM calmodulin, and 1 mg/ml bovine serum albumin. HPLC-purified γ-subunit was diluted 10-fold into this reaction mixture to effect reactivation. Reactivation was carried out at 0 °C for 18 hours.

Antibody production and purification. Monospecific polyclonal antibodies were raised in rabbits against the synthetic peptides: PhK1 (QQQNRAALFENTPKAVLFSLAEDDYG-amide), PhK5 (LRRLIDAYAFRIYGHWVKKGQQQNRG-amide), PhK9 (RRVKPVTREIVIRDPYALRPLRRLIG-amide), and PhK13 (GKFKVICLTVLASVRIYYQYRRVKPG-amide). Peptides were synthesized by standard solid-phase methods, purified, and characterized as described by Dasgupta et al. (15). Antibody production and purification was performed as previously described (16). Briefly, each antiserum was treated with sodium dextran sulfate and subjected to ammonium sulfate fractionation prior to affinity purification using Sepharose-immobilized phosphorylase kinase holoenzyme. The affinity columns used for purification of anti-PhK1 and anti-PhK5 were preequilibrated with buffer containing 50 mM MOPS (3-[N-Morpholino]propane sulfonic acid]), pH 7.0, 1 mM dithiothreitol, and 1 μM leupeptin. The columns used for anti-PhK9 and anti-PhK13 were preequilibrated with the above buffer containing 200 μM $CaCl_2$. Anti-peptide antibodies were applied to the affinity columns in their respective equilibration buffers. Following application of antibody, the affinity columns were washed with the appropriate equilibration buffer until the absorbance (280 nm) of the column effluent was less than 0.05. The anti-PhK1 and anti-PhK5 antibodies were eluted from their respective columns with 500 mM $MgCl_2$; anti-PhK9 and anti-PhK13 antibodies were eluted with 50 mM MOPS, pH 7.0, 1 mM dithiothreitol, 1 μM leupeptin, and 2 mM EDTA. Eluted antibodies were desalted using a Bio-Gel P6 DG desalting gel (Bio-Rad) and stored in phosphate-buffered saline at 4 °C. Antibody concentrations were determined spectrophotometrically using a $E_{280\ nm,\ 10\ mg/ml}$ value of 15 for IgG (18). Holoenzyme affinity columns were regenerated between use by applying 0.1 M borate, 0.5 M NaCl, pH 8.3, followed by 0.1 M acetic acid, 0.5 M NaCl, pH 4.0. Columns were stored in 50 mM MOPS, 2 mM EDTA, pH 7.0, at 4 °C between use.

Enzyme-linked immunosorbent assay (ELISA). Antipeptide antibody binding to various forms of the γ-subunit was analyzed using an indirect antibody ELISA procedure. All reagents used, except antigen and primary antibodies, were part of the ELISAmate kit (Kirkegaard & Perry Laboratories, Inc.) and the procedures used were essentially those recommended by the kit manufacturer. Polystyrene plates (96-well, flat-bottom, Corning) were coated for 1 hour at room temperature with 100 μL/well phosphorylase kinase holoenzyme (2.5 nM), γ-subunit (3 nM), or γ-δ complex (3 nM). After coating, the plate was emptied and the remaining protein binding sites were blocked with 1% bovine serum albumin. The antipeptide antibody to be analyzed was serially diluted in 1% bovine serum albumin, added to the appropriate wells, and allowed to bind for 1 hour. The plate was then emptied and washed three times with 0.02% Tween-20 (v:v). The secondary antibody (peroxidase-labeled goat anti-rabbit IgG) was then added (0.3 μg/ml) and incubated for 1 hour. The plate was emptied and washed three times, with the last wash being allowed to remain on the plate for 5 minutes. The bound secondary antibody was detected using 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide. The plate was read in kinetic mode on a Molecular Devices UVmax microplate reader at 650 nm. The microplate reader analysis software, SOFTmax (v2.01), was used for data acquisition and analysis. The maximal rate of absorbance change for each well was determined using the SOFTmax program and expressed as mOD/min. Each sample condition was run in quadruplicate.

RESULTS

Screening the four crude γ-subunit antipeptide antisera for their ability to bind phosphorylase kinase holoenzyme indicated that only anti-PhK1 bound with relatively high titer and low non-specific binding (data not shown). To reduce non-specific binding, each of the four anti-peptide antisera was subjected to affinity chromatography using Sepharose-immobilized phosphorylase kinase holoenzyme. The rationale for using immobilized holoenzyme was to selectively purify antibodies capable of binding the native γ-subunit regulatory domain. Initially, each of the antisera was applied to the holoenzyme affinity column in the presence of Ca^{++} (200 μM) and eluted with EDTA (2 mM). This was used to identify and purify antibodies capable of binding the holoenzyme in a Ca^{++}-dependent manner. Both anti-PhK9 and anti-PhK13 antibody could be purified using this approach indicating that the regions corresponding to PhK9 and PhK13 became more accessible to their respective antibodies in the presence of Ca^{++}. The antibodies raised against the more C-terminal sequences, anti-PhK1 and anti-PhK5, could not be eluted by adding EDTA, indicating that Ca^{++}-dependent conformational changes in the holoenzyme had little or no effect on the binding of these antibodies to their respective epitopes on the γ-subunit. Anti-PhK1 and anti-PhK5 could be effectively eluted from the holoenzyme affinity column using buffer containing 500 mM $MgCl_2$. For all of the studies presented here, anti-PhK9 and anti-PhK13 were routinely purified by Ca^{++}-dependent holoenzyme affinity chromatography, whereas anti-PhK1 and anti-PhK5 were purified by elution from the holoenzyme affinity column using 500 mM $MgCl_2$.

Using ELISA analysis, the four affinity-purified antibodies were compared to one another with regard to binding phosphorylase kinase holoenzyme, γ-δ complex, and the γ-subunit. These data are shown as a composite figure (Fig. 1) to facilitate comparison between the four antibodies. Anti-PhK1 bound strongly to each form of the γ-subunit, with the tightest binding to the free γ-subunit and weakest binding to the γ-δ complex. There was no evidence of Ca^{++}-dependent binding of anti-PhK1 to either the holoenzyme or the

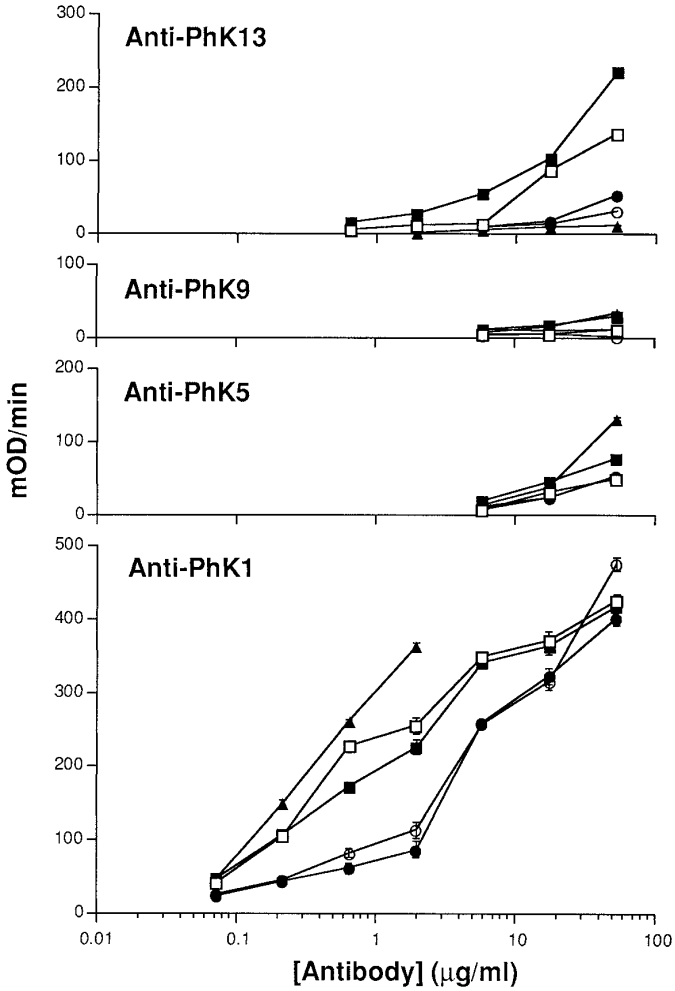

FIG. 1. Binding of affinity-purified anti-peptide antibodies to phosphorylase kinase holoenzyme (squares), γ-δ complex (circles), and the γ-subunit (triangles) in the presence of Ca^{++} (filled symbols) or EGTA (open symbols) as determined by ELISA. The details of the ELISA are described under Materials and Methods. Each point represents the mean of three independent assays (each with quadruplicate wells) ± S.D.

γ-δ complex, consistent with data from affinity chromatography of anti-PhK1. Anti-PhK5 also bound all three forms of the γ-subunit, but much less tightly than did anti-PhK1. Anti-PhK5 binding to the isolated γ-subunit appeared to be marginally tighter than its binding to the holoenzyme or γ-δ complex. Binding of anti-PhK5 to the holoenzyme, but not the γ-δ complex, was slightly enhanced by Ca^{++}. Because 2 mM EDTA did not cause significant elution of anti-PhK5 from Sepharose-immobilized holoenzyme, it is likely that the Ca^{++}-dependent changes in affinity of anti-PhK5 for the holoenzyme are rather modest. Of the four antipeptide antibodies, anti-PhK9 showed the weakest binding to the three forms of the γ-subunit. The very weak binding made it difficult to assess Ca^{++}-dependence using ELISA analysis, but there appeared to be evidence of tighter binding of anti-PhK9 to the holoenzyme and γ-δ complex in the presence of Ca^{++}, consistent with anti-PhK9 being eluted from Sepharose-immobilized holoenzyme by EDTA. Anti-PhK13 showed large differences in its binding to various forms of the γ-subunit, binding most tightly to the holoenzyme in the presence of Ca^{++} and not binding the isolated γ-subunit at all. Binding of anti-PhK13 to the γ-δ complex was significantly reduced compared to the holoenzyme. Ca^{++}-dependent binding of anti-PhK13 to the holoenzyme was very marked, consistent with the antibody's behavior on Sepharose-immobilized holoenzyme, but the Ca^{++}-dependence of binding to the γ-δ complex was less pronounced. The binding data for anti-PhK13 indicate that this affinity-purified antibody probably recognizes a conformational epitope that is presented on the surface of the holoenzyme, but which is either not formed or not presented in the isolated γ-subunit. Interactions of the α-, β-, and δ-subunits appear to be important in forming this conformational epitope since there are large differences in anti-PhK13 binding to the γ-subunit depending upon whether Ca^{++} and the α-, β-and δ-subunits are present.

DISCUSSION

It is interesting to compare the results of the present study with results obtained in a complementary investigation where affinity-purified anti-PhK1, anti-PhK5, anti-PhK9, and anti-PhK13 were analyzed for the ability to activate or inhibit phosphorylase kinase catalytic activity (16). In their effects on catalytic activity, anti-PhK1 and anti-PhK5 were similar to each other in several respects, as were anti-PhK9 and anti-PhK13. Both anti-PhK1 and anti-PhK5 markedly activated the catalytic activity of the holoenzyme and the γ-δ complex, in the presence and absence of Ca^{++}. In the present study, anti-PhK1 and anti-PhK5 both bound Sepharose-immobilized holoenzyme in a Ca^{++}-independent manner and bound the isolated γ-subunit tighter than other forms of the γ-subunit. In contrast, anti-PhK9 and anti-PhK13 both bound Sepharose-immobilized holoenzyme in a Ca^{++}-dependent manner and were both inhibitory with regard to their effects on catalytic activity.

The data from work with antipeptide antibodies, as well as synthetic γ-subunit regulatory domain peptides, can be used to develop a description of the structure and function of each region within the γ-subunit regulatory domain. The very C-terminus of the γ-subunit, corresponding to the sequence of PhK1, does not itself bind the δ-subunit (15) or have pseudosubstrate/autoinhibitory activity (19), but appears to be tightly linked to such a region because anti-PhK1 binding is capable of markedly activating the catalytic activity of the holoenzyme and γ-δ complex (16). The region corresponding to PhK5, which is immediately N-termi-

nal to PhK1, appears to interact directly with the δ-subunit (15) and function as a pseudosubstrate/autoinhibitory domain (19). The structure and interactions of the PhK5 region are likely to be affected by antibody binding anywhere along the C-terminal 45 residues of the γ-subunit since both anti-PhK1 and anti-PhK5 antibodies have qualitative similar effects on catalytic activity (16). Neither anti-PhK1 nor anti-PhK5 binding to the holoenzyme is significantly altered by Ca^{++} (Fig. 1), indicating that even though PhK5 binds the δ-subunit, Ca^{++}-dependent changes in the δ-subunit do not significantly alter the presentation of the PhK1 or PhK5 regions on the surface of the holoenzyme. However, important interactions between the δ-subunit and these regions of the γ-subunit are indicated by the fact that anti-PhK1 and anti-PhK5 require Ca^{++} to achieve maximal stimulation of holoenzyme and γ-δ complex catalytic activity (16). Similarly, important functional interactions of the PhK1 and PhK5 regions of the γ-subunit with the α- and β-subunits are suggested by data indicating that anti-PhK1 and anti-PhK5 stimulate the catalytic activity of the holoenzyme to a much greater extent than the γ-δ complex (16). Additional evidence indicating important interactions between the PhK1 and PhK5 regions of the γ-subunit and the α- and β-subunits comes from data showing that cAMP-dependent protein kinase-catalyzed phosphorylation of the holoenzyme can completely block holoenzyme activation by both anti-PhK1 and anti-PhK5 (16).

Antipeptide antibodies raised against PhK9 and PhK13 have also been useful in identifying interactions between the γ-subunit and other subunits in the holoenzyme complex. Both anti-PhK9 and anti-PhK13 were affinity-purified in a Ca^{++}-dependent manner indicating that a Ca^{++}-dependent conformational change in the δ-subunit alters the presentation of the PhK9 and PhK13 regions on the surface of the holoenzyme. PhK13, but not PhK9, has previously been shown to bind the δ-subunit (calmodulin) with high affinity (15). Interestingly, affinity-purified anti-PhK13 does not bind the isolated γ-subunit (Fig. 1), indicating the importance of the δ-subunit in properly presenting the PhK13 region for recognition by affinity-purified anti-PhK13. Although the PhK9 region does not appear to directly interact with the δ-subunit (15), its close proximity to the PhK13 region probably explains why its structure is altered when the holoenzyme binds Ca^{++}. Further evidence that conformational changes in the PhK9 and PhK13 regions are tightly linked is provided by data showing that both anti-PhK9 and anti-PhK13 are capable of inhibiting holoenzyme and γ-δ complex catalytic activity (16). Anti-PhK9 effects on catalytic activity are probably due to indirect conformational effects on the PhK13 region, since the PhK13 region, but not the PhK9 region, has been identified as a pseudosubstrate/autoinhibitory domain (19, 20).

Important interactions between the PhK13 region of the γ-subunit and the α- and β-subunits are indicated by several observations made using anti-PhK13. One indication is that anti-PhK13 binding to the holoenzyme is significantly stronger than binding to the γ-δ complex or the free γ-subunit (Fig. 1). Another is that marked Ca^{++}-dependent binding of anti-PhK13 to the γ-subunit occurs with the holoenzyme, but not with the γ-δ complex (Fig. 1). Additional evidence that the α- and β-subunits interact with the PhK13 region is provided by kinetic studies of anti-PhK13 inhibition of catalytic activity (16). Anti-PhK13 is a partial noncompetitive antagonist of the holoenzyme, but a full antagonist of the γ-δ complex (16). Thus, the α- and β-subunits appear to prevent anti-PhK13 from fully inhibiting the catalytic activity of the holoenzyme.

In summary, affinity-purified antipeptide antibodies raised against the regulatory domain of the γ-subunit are useful probes for detecting structural changes in the γ-subunit resulting from its interactions with other subunits. These are the first studies to show that the conformation of the γ-subunit regulatory domain is altered by its interactions with the α-, β- and γ-subunits and indicate that more detailed studies focused on the role of the γ-subunit regulatory domain in these subunit interactions are warranted.

ACKNOWLEDGMENTS

This work was supported by grants to D.K.B. from NIH (GM39290) and the American Heart Association, National. The authors also thank Bahareh Patrick, Theresa Honeycutt, and Dr. Glenn E. Meixell for their valuable technical assistance and advice.

REFERENCES

1. Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., and Nairn, A. (1978) *FEBS Lett.* **92,** 287–293.
2. Cohen, P. (1973) *Eur. J. Biochem.* **34,** 1–14.
3. Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973) *Biochemistry* **12,** 567–573.
4. Pickett-Gies, C. A., and Walsh, D. A. (1986) *in* The Enzymes (Boyer, P. D., and Krebs, E. G., Eds.), Vol. XVII, pp. 395–459, Academic Press, New York.
5. Hayakawa, T., Perkins, J. P., and Krebs, E. G. (1973) *Biochemistry* **12,** 574–580.
6. Chan, K.-F. J., and Graves, D. J. (1982) *J. Biol. Chem.* **257,** 5956–5961.
7. Kee, S. M., and Graves, D. J. (1986) *J. Biol. Chem.* **261,** 4732–4737.
8. Reimann, E. M., Titani, K., Ericsson, L. H., Wade, R. D., Fischer, E. H., and Walsh, K. A. (1984) *Biochemistry* **23,** 4185–4192.
9. Skuster, J. R., Chan, K.-F. J., and Graves, D. J. (1980) *J. Biol. Chem.* **255,** 2203–2210.
10. Picton, C., Klee, C. B., and Cohen, P. (1980) *Eur. J. Biochem.* **111,** 553–561.
11. Lambooy, P. K., and Steiner, R. F. (1982) *Arch. Biochem. Biophys.* **213,** 551–556.
12. Harris, W. R., Malencik, D. A., Johnson, C. M., Carr, S. A., Roberts, G. D., Byles, C. A., Anderson, S. R., Heilmeyer, L. M. G.,

Fischer, E. H., and Crabb, J. W. (1990) *J. Biol. Chem.* **265,** 11740–11745.
13. Cox, S., and Johnson, L. N. (1992) *Protein Eng.* **5,** 811–819.
14. Huang, C.-Y. F., Yuan, C.-J., Livanova, N. B., and Graves, D. J. (1993) *Mol. Cell. Biochem.* **127/128,** 7–18.
15. Dasgupta, M., Honeycutt, T., and Blumenthal, D. K. (1989) *J. Biol. Chem.* **264,** 17156–17163.
16. Wangsgard, W. P., Meixell, G. E., Dasgupta, M., and Blumenthal, D. K. (1996) *J. Biol. Chem.* **271,** 21126–21133.
17. Crabb, J. W., and Heilmeyer, L. M. G., Jr. (1984) *J. Biol. Chem.* **259,** 6346–6350.
18. Fasman, G. D. (Ed.) (1989) Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Boca Raton, FL.
19. Dasgupta, M., and Blumenthal, D. K. (1995) *J. Biol. Chem.* **270,** 22283–22289.
20. Huang, C.-Y. F., Yuan, C.-J., Blumenthal, D. K., and Graves, D. J. (1995) *J. Biol. Chem.* **270,** 7183–7188.