Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5222–5226, June 1991
Biochemistry

Phosphorylation of protein 4.1 on tyrosine-418 modulates its function *in vitro*

(epidermal growth factor receptor/spectrin/actin assembly)

GOSUKONDA SUBRAHMANYAM*, PAUL J. BERTICS†‡, AND RICHARD A. ANDERSON*‡§

Department of *Pharmacology and †Physiological Chemistry, and the ‡Cell and Molecular Biology Program, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706

Communicated by Vincent T. Marchesi, March 15, 1991

ABSTRACT **Protein 4.1 was initially characterized as a protein that regulates cytoskeletal assembly in erythrocytes. However, recent studies have shown that protein 4.1 is ubiquitous in mammalian cells. Here, we show that protein 4.1 is phosphorylated on tyrosine by the epidermal growth factor receptor (EGFR) tyrosine kinase. The phosphorylation site has been localized to the 8-kDa domain, which has one tyrosine, tyrosine-418. The 8-kDa region is required for the assembly of the spectrin/actin complex, and phosphorylation by EGFR reduced the ability of protein 4.1 to promote the assembly of the spectrin/actin/protein 4.1 ternary complex. Immunoblotting with anti-phosphotyrosine antibodies showed that purified protein 4.1 contained phosphorylated tyrosine, and this increased upon phosphorylation by EGFR. This suggests that tyrosine phosphorylation of protein 4.1 occurs *in vivo* and may be functionally significant. The tyrosine phosphorylation site is in the center of a sequence motif that is expressed by a differentiation-specific splicing mechanism.**

The human erythrocyte membrane skeleton is composed of a network of proteins that regulate cell shape and deformability. The membrane skeleton also gives the erythrocytes their mechanical integrity required for passage through the microcirculation. The major components of this network are spectrin and actin, which form the lattice structure (for review, see ref. 1). Protein 4.1 regulates the association between spectrin and actin (2) and attaches the spectrin/actin/protein 4.1 complex to the overlying membrane by an interaction between protein 4.1 and the integral membrane glycophorins (3–5). The interaction between protein 4.1 and the glycophorins is tightly regulated and requires phosphatidylinositol 4,5-bisphosphate as an obligate cofactor (3, 5). Protein 4.1 also associates with protein band 3, but this is of lower affinity and does not occur when protein 4.1 is bound to spectrin (6).

Protein 4.1 is cleaved by α-chymotrypsin into four resistant domains: the 30-kDa, 16-kDa, 8-kDa, and 24-kDa regions (7). The function of two of these domains is well defined. The 30-kDa region is involved in binding to both glycophorin and band 3 (3, 7). The 8-kDa region promotes the assembly of the spectrin/actin network (8). The 8-kDa region of protein 4.1 also interacts with myosin and regulates myosin ATPase activity *in vitro* (9). These observations suggest that protein 4.1 is a multifunctional molecule.

Protein 4.1 is a phosphoprotein *in vivo* and a substrate for cAMP-dependent protein kinase and protein kinase C (10–12). *In vitro* phosphorylation of protein 4.1 by membrane-associated casein kinase I (13), protein kinase C, and cAMP-dependent kinase (12) reduces protein 4.1 binding to spectrin. Phosphorylation of protein 4.1 by the latter two kinases reduces its ability to promote the assembly of the spectrin/actin/protein 4.1 complex (12). However, phosphorylation of protein 4.1 by protein kinase C also reduces protein 4.1 binding to band 3 but not to glycophorin, while phosphorylation by cAMP-dependent kinase does not affect membrane interactions (12). These results suggest that protein 4.1 is phosphorylated at multiple sites by different protein kinases, and each phosphorylation event selectively modulates protein 4.1 function. The phosphorylation sites by cAMP-dependent protein kinase and protein kinase C are in the 8-kDa and 16-kDa domains (10).

Protein 4.1 isoforms have been identified in a wide variety of cells (ref. 14 and references cited therein), and the diversity in these protein 4.1 molecules is partly due to alternative splicing of mRNA (15–17). The erythroid form of protein 4.1 contains a 21-amino acid sequence (amino acids 407–427) that is inserted by a lineage-specific splicing mechanism during terminal differentiation (17). This sequence motif (motif I) may be required for spectrin/actin/protein 4.1 assembly and contains a regulatory sequence that is a site for tyrosine phosphorylation. We report that phosphorylation on tyrosine-418, in motif I, functionally modulates the ability of protein 4.1 to assemble spectrin and actin.

MATERIALS AND METHODS

Materials. Human blood was obtained fresh from the American Red Cross Society of Wisconsin. Fast-flow S-Sepharose was obtained from Pharmacia, DE-52 from Whatman, Sepharose CL-4B from Sigma, and inorganic [^{32}P]phosphate and sodium [^{125}I]iodide from DuPont and Amersham. [γ-^{32}P]ATP was synthesized using a [^{32}P]ATP synthesis kit from Promega; monoclonal anti-phosphotyrosine antibodies (PY-20 antibodies) were from ICN; nitrocellulose was from Schleicher & Schuell. Protein A, α-chymotrypsin [treated with "N^{α}-(tosyl)-L-lysine chloromethyl ketone" (7-amino-1-chloro-3-tosylamido-2-heptanone)], and trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) were from Sigma; all other reagents were from Sigma.

Purification of Proteins. Protein purification was carried out on ice or at 4°C, unless otherwise noted. Protein 4.1 was extracted from inside-out erythrocyte membrane vesicles with 2.5 M urea/1 M KCl/0.1 M glycine, pH 7.7/5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride on ice. The extract was dialyzed extensively against 5 mM sodium phosphate, pH 7.7/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (buffer A) and applied to a DE-52 column (7 cm × 1 cm) preequilibrated with 20 mM KCl in buffer A. Protein 4.1 was eluted from the column with a linear salt gradient of 20–400 mM KCl

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGFR, epidermal growth factor receptor; BSA, bovine serum albumin.

§To whom reprint requests should be addressed at: Department of Pharmacology, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706.

in buffer A. Fractions containing protein 4.1 were pooled, adjusted to pH 7.0 and 200 mM KCl in buffer A, and chromatographed on an S-Sepharose column (5 cm × 1 cm). Protein 4.1 was eluted from the column with a linear salt gradient of 200–800 mM KCl in buffer A. Protein 4.1 fractions were pooled and concentrated by dialysis against 25% (wt/vol) polyethylene glycol (M_r 8000)/10 mM Tris Cl, pH 7.4/20 mM NaCl/130 mM KCl (isotonic KCl buffer) and stored on ice. Spectrin was extracted from erythrocyte ghosts with 0.1 mM EDTA (pH 8.0) at 37°C in the presence of 0.1 mM diisopropyl fluorophosphate and purified further on a Sepharose CL-4B gel filtration column (18). Spectrin dimers were concentrated as above and stored in isotonic KCl buffer with 50% glycerol at −20°C. Actin was purified from chicken muscle acetone powder (19). Epidermal growth factor receptor (EGFR) tyrosine kinase was affinity-purified from solubilized membranes of A-431 human epidermoid carcinoma cells, as described (20). All proteins were analyzed for purity by SDS/PAGE.

Phosphorylation of Protein 4.1 by EGFR Kinase. Phosphorylation reactions were carried out in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4/5 mM $MgCl_2$/4 mM $MnCl_2$/1 mM dithiothreitol/0.1 mM orthovanadate/130 mM KCl/0.1 mM [γ-^{32}P]ATP (500–800 cpm/pmol) with 5–10 pmol of EGFR per 1–2 μmol of protein 4.1 for 1 hr at 20°C. Phospho amino acid analysis was carried out as described (21). Two-dimensional tryptic phosphopeptide mapping was done according to Gould and Hunter (22).

Partial Proteolysis of Protein 4.1. Protein 4.1 was subjected to limited proteolysis with α-chymotrypsin on ice to generate the characteristic peptide fragments (7).

Sedimentation Assay for Spectrin/Actin/Protein 4.1 Complex. Assembly of the spectrin/actin/protein 4.1 ternary complex was carried out exactly as described (23), with protein 4.1 ranging from 1 to 6 μg. The spectrin/actin/protein 4.1 complex was sedimented by centrifugation at 150,000 × *g* for 30 min. For quantitative studies, the supernatant and pellet were analyzed by SDS/7–15% PAGE and the proteins were stained with Coomassie blue. The dye bound to the proteins was extracted with aqueous 25% pyridine and the absorbance was measured at 605 nm (24). In each gel, known concentrations of bovine serum albumin (BSA) were run as standards, and the amount of spectrin in the supernatant and in the pellet was determined.

For determining phosphate incorporation and its effect on ternary complex formation, 120 μg of protein 4.1 was incubated with EGFR in the presence of [γ-^{32}P]ATP for up to 1 hr at 30°C. At the end of 5, 10, 15, 20, 30, 40, and 60 min of incubation, aliquots (5 μg) of protein 4.1 were assayed for their ability to promote spectrin/actin assembly and for ^{32}P incorporation. For these experiments, the spectrin/actin assembly buffer contained the same specific activity of [γ-^{32}P]ATP as for phosphorylation reactions. The results showed that there was no further incorporation of ^{32}P during spectrin/actin assembly.

Immunoblotting with Anti-Phosphotyrosine Antibodies. Protein 4.1 (5 μg) was incubated with ATP in the presence or absence of EGFR for 1 hr at 25°C. At the end of the incubation period, protein samples were run in SDS/7–15% polyacrylamide gradient gels and transferred to a nitrocellulose sheet by standard procedures (25). Nonspecific binding sites were blocked by incubating the nitrocellulose sheet with 5% BSA and 0.25% gelatin for 24 hr in phosphate-buffered saline (PBS) containing 0.05% Tween 20. The blot was then incubated with PY-20 antibodies (1:500 dilution in PBS with 3% BSA) for 4 hr. Where competition with phospho amino acids was carried out, identical blots were incubated with antibody in the absence or presence of 10 mM phosphotyrosine and 10 mM *p*-nitrophenyl phosphate. The blots were washed with PBS and incubated for 1 hr with ^{125}I-labeled protein A (3 × 10^7 cpm/μg). The blots were washed several times with PBS and then exposed to x-ray film at −80°C.

Other Analytical Techniques. The protein concentration during purification of spectrin, actin, and protein 4.1 was assayed according to Lowry *et al.* (26). SDS/PAGE was according to Laemmli (27).

RESULTS

Tyrosine Phosphorylation of Protein 4.1. Protein 4.1 purified as described in *Materials and Methods* did not contain contaminating protein kinase activity. Protein 4.1 was incubated with or without EGFR in the presence of [γ-^{32}P]ATP. Fig. 1*A* shows the Coomassie blue-stained SDS/polyacrylamide gel and Fig. 1*B* the corresponding autoradiograph demonstrating that protein 4.1 was readily phosphorylated by the EGFR kinase. To show that protein 4.1 was phosphorylated on tyrosine residues, the purified protein 4.1 was immunoblotted with anti-phosphotyrosine antibodies in the presence or absence of EGFR. Purified protein 4.1 was, to some extent, phosphorylated on tyrosine, and the level of phosphotyrosine was increased upon phosphorylation by EGFR (Fig. 1*C*). The antibody reactivity toward phosphorylated protein 4.1 and EGFR was completely blocked by 10 mM phosphotyrosine and 10 mM *p*-nitrophenyl phosphate. These antibodies did not crossreact nonspecifically with any proteins in erythrocyte membrane "ghosts" (results not shown). The phosphorylated protein 4.1 band was cut from the gel and analyzed for phospho amino acids. One-dimensional phospho amino analysis showed only phosphotyrosine (Fig. 1*D*). Stoichiometric analysis of tyrosine phosphorylation showed up to 0.6 mol of phosphate incorporated per mol of protein 4.1 (data not shown).

Localization of the Tyrosine Phosphorylation Site in Protein 4.1. When protein 4.1 was partially digested with α-chymotrypsin, it gave characteristic peptide fragments (7). Under the same conditions, the phosphorylation sites within protein 4.1 have been determined. Both the 30-kDa and 8-kDa fragment appeared to contain phosphorylation sites (Fig. 2). When the time course of phosphorylation was coupled with limited proteolysis, the 8-kDa region was the preferred site for phosphorylation (results not shown). Since the 30-kDa fragment may be contaminated with the 32-kDa fragment, which contains the 8-kDa region, two-dimensional tryptic phosphopeptide mapping was done to determine whether there were two phosphorylation sites. The two-dimensional phosphopeptide maps of intact protein 4.1 and the 30-kDa and 8-kDa fragments were identical, suggesting that only the 8-kDa region was phosphorylated by EGFR (Fig. 3). When

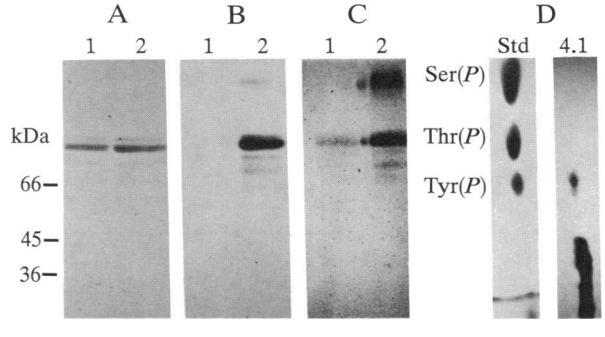

FIG. 1. Tyrosine phosphorylation of protein 4.1 by EGFR. Protein 4.1 (4 μg) was incubated with [γ-^{32}P]ATP in the absence (lanes 1) or presence (lanes 2) of EGFR. (*A*) Coomassie blue-stained gel. (*B*) Corresponding autoradiograph. (*C*) Western immunoblot probed with anti-phosphotyrosine antibodies (PY-20 antibodies). (*D*) Phospho amino acid analysis of phosphorylated protein 4.1. Left lane, phospho amino acid standards; right lane, autoradiograph of [^{32}P]phospho amino acids from protein 4.1.

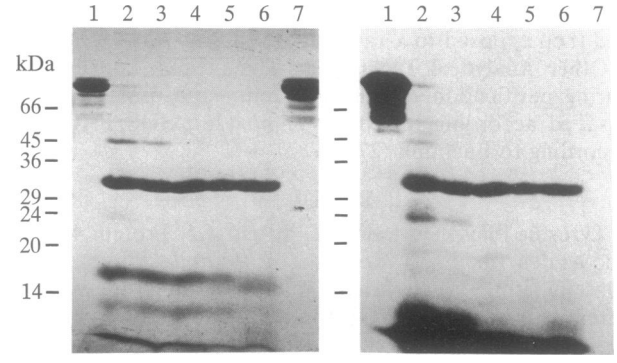

FIG. 2. Localization of the site of tyrosine phosphorylation by limited proteolysis of protein 4.1. Protein 4.1 (40 μg) was incubated with or without EGFR for 1 hr. The phosphorylated form of protein 4.1 was incubated on ice for 30 min at chymotrypsin/protein 4.1 ratios ranging from 1:400 to 1:25. Lanes 1, no chymotrypsin; lanes 2, 1:400; lanes 3, 1:200; lanes 4, 1:100; lanes 5, 1:50; lanes 6, 1:25; lanes 7, without chymotrypsin in the absence of added EGFR. Proteolytic fragments were separated by SDS/7–15% gradient PAGE in the presence of SDS. (*Left*) Coomassie blue-stained gel. (*Right*) Corresponding autoradiograph of the gel.

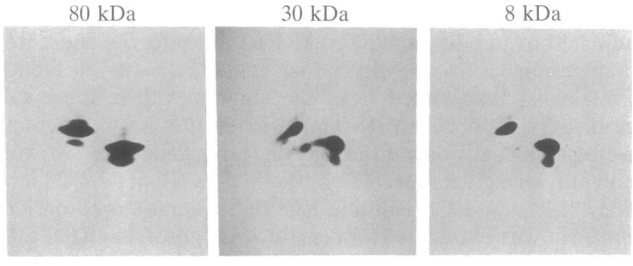

FIG. 3. Tyrosine phosphorylation occurs in the 8-kDa domain. Two-dimensional tryptic phosphopeptide maps of intact protein 4.1 (80 kDa), the 30-kDa peptide, and the 8-kDa peptide are shown.

extensive proteolysis of phosphorylated protein 4.1 was carried out with α-chymotrypsin, the amount of radioactive label from the 30-kDa region was reduced, suggesting that the 8-kDa region was the major phosphorylation site (data not shown). Since the 8-kDa region has only one tyrosine residue, tyrosine-418 (8, 28), this must be the phosphate acceptor. The stoichiometry of phosphate incorporated per mole of protein 4.1 also suggests only one phosphorylation site within protein 4.1.

Tyrosine Phosphorylation Blocks Promotion of Spectrin/Actin Assembly. Under physiological ionic strength, the 8-kDa fragment of protein 4.1 promotes assembly of the spectrin/actin/protein 4.1 ternary complex (23). To study the effect of tyrosine phosphorylation on ternary complex formation, protein 4.1 was incubated with ATP in the presence or absence of the EGFR. Phosphorylation of protein 4.1 by EGFR reduced its ability to promote the ternary complex formation by 50–70%. The reduction in the ternary complex formation was proportional to the extent of phosphorylation of protein 4.1 by EGFR (Fig. 4). Incubation of protein 4.1 with EGFR in the absence of ATP did not effect the formation of ternary complex (data not shown). The maximal stoichiometry of phosphorylation appeared to be about 0.6 mol of phosphate per mol of protein 4.1, which corresponds well with the extent of functional modification. These results demonstrate that phosphorylation of tyrosine-418 regulates protein 4.1 function.

When phosphorylation of protein 4.1 was attempted within the spectrin/actin/protein 4.1 complex, the phosphorylation site was not accessible to EGFR. However, the presence of spectrin or actin alone did not inhibit phosphorylation of protein 4.1 by EGFR, providing further evidence for phosphorylation of largely the 8-kDa region (Fig. 5).

DISCUSSION

Protein tyrosine phosphorylation has been implicated as a regulatory event important for cell growth, differentiation, and many other cellular events. In this paper, it is shown that tyrosine phosphorylation of the human erythroid protein 4.1 by EGFR modulates its function. The phosphorylation site has been localized to tyrosine-418, within the 8-kDa domain.

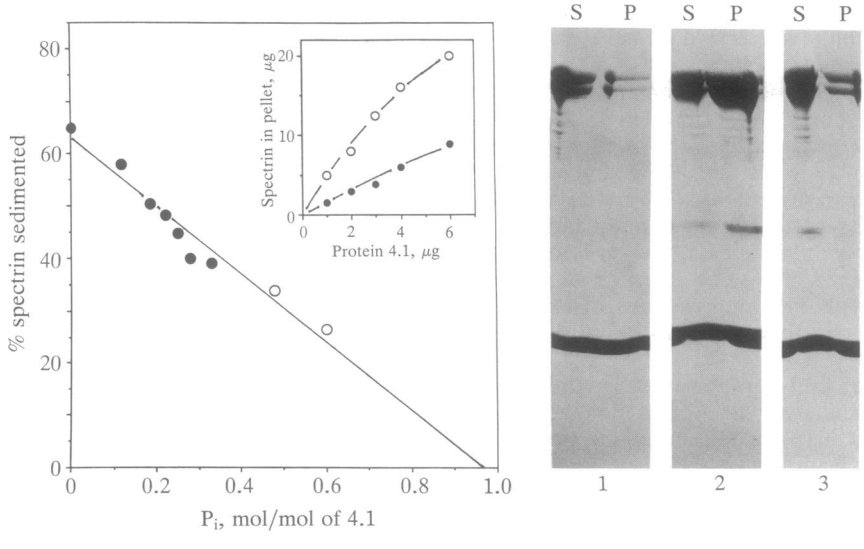

FIG. 4. Tyrosine phosphorylation of protein 4.1 blocks assembly of the spectrin/actin/protein 4.1 ternary complex. (*Left*) Protein 4.1 was phosphorylated with EGFR for various times to determine the molar phosphate incorporation. At each time point, 5 μg of protein 4.1 was assayed for its ability to promote spectrin/actin assembly, and the percentage of spectrin sedimented was calculated. Results obtained from a single experiment (●) and from two different phosphorylation reactions (○) are shown. In these experiments protein 4.1 was phosphorylated for 1 hr with EGFR at 20°C and then assayed for spectrin/actin assembly-promoting activity. (*Inset*) Concentration dependence of phosphorylated protein 4.1 (●) and unphosphorylated protein 4.1 (○) in promoting spectrin/actin assembly. In this experiment, 0.6 mol of phosphate was incorporated per mol of protein 4.1. (*Right*) SDS/PAGE analysis of spectrin/actin complex sedimented in the absence of protein 4.1 (lanes 1), in the presence of 4 μg of protein 4.1 (lanes 2), or in the presence of 4 μg of the phosphorylated form of protein 4.1 (lanes 3). Equal volumes from supernatant (lanes S) and pellet (lanes P) were loaded in each lane.

Table 1. Comparison of amino acid sequences of tyrosine phosphorylation sites

Protein	Sequence surrounding tyrosine																Ref.
	−9	−8	−7	−6	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5	+6	+7	
Protein 4.1	Arg	Glu	**Arg**	Leu	Asp	Gly	Glu	Asn	Ile	Tyr	Ile	Arg	**His**	Ser	Asn	Leu	Met	28
p120$^{gag-abl}$	Leu	Ser	**Arg**	Leu	Met	Thr	Gly	Asp	Thr	Tyr	Thr	Ala	**His**	Ala	Gly	Ala	**Lys**	29
pp60^{v-src}	Leu	Ala	**Arg**	Leu	Ile	Glu	Asp	Asn	Glu	Tyr	Thr	Ala	**Arg**	Gln	Gly	Ala	**Lys**	30
p90$^{gag-yes}$	Leu	Ala	**Arg**	Leu	Ile	Glu	Asp	Asn	Glu	Tyr	Thr	Ala	**Arg**	Gln	Gly	Ala	**Lys**	31
p140$^{gag-fps}$	Met	Ser	**Arg**	Gln	Glu	Glu	Asp	Gly	Val	Tyr	Ala	Ser	Thr	Gly	Gly	Met	**Lys**	32
p110$^{gag-fes}$	Met	Ser	**Arg**	Glu	Ala	Ala	Asp	Gly	Ile	Tyr	Ala	Ala	Ser	Gly	Gly	Leu	**Arg**	33

The peptide sequence shown starts from amino acid 409 of the erythroid protein 4.1 sequence (28). Boldface indicates the conserved basic amino acids.

Three lines of evidence are presented for localization of the phosphorylation site: (*i*) proteolysis with α-chymotrypsin showed the 8-kDa peptide to be the major phosphopeptide; (*ii*) two-dimensional tryptic phosphopeptide maps from intact protein 4.1 and 30-kDa and 8-kDa peptides were identical; and (*iii*) phosphorylation modulates the ability of protein 4.1 to promote spectrin/actin assembly, and protein 4.1 in the spectrin/actin/protein 4.1 complex was less accessible to the EGFR kinase.

The 8-kDa peptide has only one tyrosine residue (tyrosine-418) in its sequence (8, 28). When this sequence was compared with consensus tyrosine phosphorylation sequences, it was clear that the region around tyrosine-418 has a consensus sequence for phosphorylation by tyrosine kinases (Table 1). This 8-kDa region is important for the function of the erythroid isoform of protein 4.1 and the 21-amino acid sequence (motif I) containing the tyrosine phosphorylation site is specifically "spliced into" protein 4.1 upon differentiation from progenitor cells (17). Phosphorylation of tyrosine-418, in the center of motif I, may be the functional significance of insertion or deletion of this sequence. Potentially, tyrosine phosphorylation may be important for protein 4.1 function during erythrocyte development.

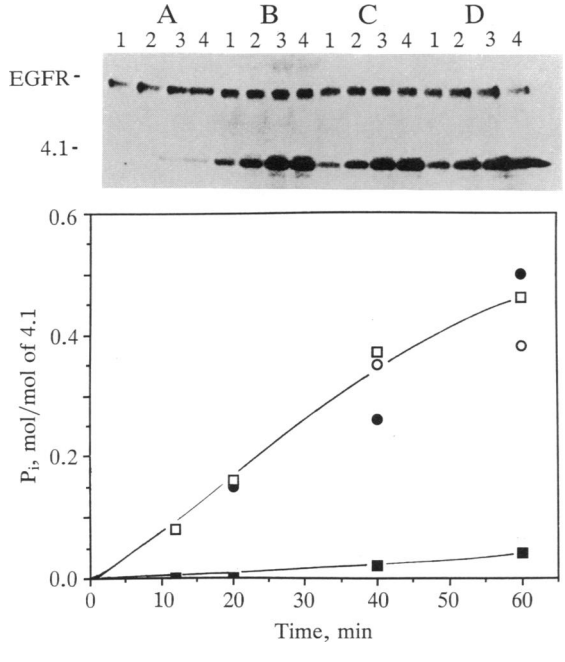

FIG. 5. Time course of phosphorylation of protein 4.1 by EGFR. (*Lower*) ■, Protein 4.1 in the ternary complex with spectrin and actin; ○, protein 4.1 and spectrin; ●, protein 4.1 and actin; □, protein 4.1 alone. (*Upper*) Autoradiograph of protein 4.1 phosphorylation in the ternary complex of spectrin/actin/4.1 (*A*), of protein 4.1 and spectrin (*B*), of protein 4.1 and actin (*C*), and of protein 4.1 alone (*D*). Lanes 1–4, time points of 12, 20, 40, and 60 min, respectively.

In addition to protein kinase A and protein kinase C, erythrocytes contain protein-tyrosine kinases and protein-tyrosine-phosphatases (34–36). The functions of these enzymes are not known at present. However, the results presented in this paper suggest that erythrocyte tyrosine kinases could play a role in the maintenance of the erythrocyte membrane skeleton. Indeed, a small fraction of protein 4.1 appears to be phosphorylated on tyrosine when isolated (Fig. 1*C*). Potentially, a protein that is phosphorylated by one protein tyrosine kinase may also be a substrate for other tyrosine kinases; for example, phosphatidylinositol 3-kinase, phospholipase C-γ, and enolase are substrates for many tyrosine kinases (37–40). The *in vitro* and *in vivo* tyrosine phosphorylation of protein 4.1 suggests that it could also be a substrate for a number of tyrosine kinases. In addition to erythrocytes, protein 4.1 isoforms are found in nonerythroid cells, at various intracellular locations (14). In the human epidermoid carcinoma cell line A-431, at least four isoforms of protein 4.1 were recognized by antibodies against erythroid protein 4.1 (unpublished results). It is to be determined whether any of these isoforms are involved in EGF-induced cytoskeletal reorganization in A-431 cells.

The search for a similar phosphorylation sequence in other regions of the protein 4.1 molecule showed another consensus sequence for phosphorylation by tyrosine kinases at tyrosine-250 in the 30-kDa region. This sequence has all of the requirements for tyrosine phosphorylation—i.e., at position −7, it has arginine; in close proximity to the amino-terminal side of tyrosine it has glutamate; and at positions +5 and +7 positions it has glycine and lysine residues, respectively. However, this sequence was not recognized by the EGFR kinase. Most protein 4.1 isoforms share homologies within the 30-kDa region, and this region also shares some homology with the cytoskeletal proteins ezrin and talin (41, 42). In A-431 cells, ezrin has been shown to be phosphorylated on tyrosine by EGFR (43). It will be interesting to find out whether this cryptic phosphorylation site in erythroid protein 4.1 is phosphorylated by other tyrosine kinases in nonerythroid forms and whether phosphorylation has functional significance.

We thank Dr. Mark Mooseker for the generous gift of acetone extract of chicken muscle. P. S. Leventhal and L. Hubler for the purification of EGFR, and Dr. C. Bazenet and Mr. G. H. Jenkins for critical reading of the manuscript. This work was supported by grants to R.A.A. from the National Institutes of Health (GM38906), March of Dimes (5-659), American Heart Association (880934), and American Cancer Society (BC-639) and to P.J.B. from the National Institutes of Health (CA47881) and March of Dimes (1-1206).

1. Marchesi, V. T. (1985) *Annu. Rev. Cell Biol.* **1**, 531–561.
2. Fowler, V. & Taylor, D. L. (1980) *J. Cell Biol.* **85**, 361–376.
3. Anderson, R. A. (1989) in *Red Blood Cell Membranes, Structure, Function, and Clinical Implications*, eds. Agre, P. & Parker, J. C. (Dekker, New York), pp. 187–236.
4. Anderson, R. A. & Lovrein, R. E. (1984) *Nature (London)* **307**, 655–658.

5. Anderson, R. A. & Marchesi, V. T. (1985) *Nature (London)* **318,** 295–298.
6. Pasternack, G. R., Anderson, R. A., Leto, T. L. & Marchesi, V. T. (1985) *J. Biol. Chem.* **260,** 3676–3683.
7. Leto, T. L. & Marchesi, V. T. (1984) *J. Biol. Chem.* **259,** 4603–4608.
8. Correas, I., Speicher, D. W. & Marchesi, V. T. (1986) *J. Biol. Chem.* **261,** 13362–13366.
9. Pasternack, G. R. & Racusen, R. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9712–9716.
10. Horne, W. C., Leto, T. L. & Marchesi, V. T. (1985) *J. Biol. Chem.* **260,** 9073–9076.
11. Ling, E., Danilov, Y. N. & Cohen, C. M. (1988) *J. Biol. Chem.* **263,** 2209–2216.
12. Danilov, Y. N., Fennell, R., Ling, E. & Cohen, C. M. (1990) *J. Biol. Chem.* **265,** 2556–2562.
13. Eder, P. S., Soong, C. J. & Tao, M. (1986) *Biochemistry* **25,** 1764–1770.
14. Anderson, R. A., Correas, I., Mazzuco, C., Castle, J. D. & Marchesi, V. T. (1988) *J. Cell. Biochem.* **37,** 269–284.
15. Tang, K. T., Qin, Z., Leto, T. L., Marchesi, V. T. & Benz, E., Jr. (1990) *J. Cell Biol.* **110,** 617–624.
16. Conboy, J. G., Chan, J., Mohandas, N. & Kan, W. Y. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9602–9605.
17. Tang, K. T., Leto, T. L., Correas, I., Alonso, M. A. & Marchesi, V. T. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3713–3717.
18. Morrow, J. S., Speicher, D. W., Knowles, W. J., Hsu, C. J. & Marchesi, V. T. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6592–6596.
19. Spudich, J. E. & Watt, S. (1971) *J. Biol. Chem.* **246,** 4866–4871.
20. Weber, W., Bertics, P. J. & Gill, G. N. (1984) *J. Biol. Chem.* **259,** 14631–14636.
21. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
22. Gould, K. L. & Hunter, T. (1988) *Mol. Cell. Biol.* **8,** 3345–3356.
23. Correas, I., Leto, T. L., Speicher, D. W. & Marchesi, V. T. (1986) *J. Biol. Chem.* **261,** 3310–3315.
24. Fenner, C., Traut, R. R., Mason, D. T. & Wikman-Coffel, J. (1975) *Anal. Biochem.* **63,** 595–602.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4353.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
27. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
28. Conboy, J. G., Kan, Y. W., Shohet, S. B. & Mohandas, N. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9512–9516.
29. Reddy, E. P., Smith, M. J. & Srinivasan, A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3623–3627.
30. Schwartz, D. E., Tizard, R. & Gilbert, W. (1983) *Cell 32,* 853–869.
31. Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y. & Yoshida, M. (1982) *Nature (London)* **297,** 205–208.
32. Shibuya, M. & Hanafusa, H. (1982) *Cell* **30,** 787–795.
33. Hampe, A., Laprevotte, I., Galibert, F., Fedele, L. A. & Scherr, C. (1982) *Cell* **30,** 775–785.
34. Dekowski, S. A., Rybicki, A. & Drickamer, K. (1983) *J. Biol. Chem.* **258,** 2750–2753.
35. Mohamed, A. H. & Steck, T. L. (1986) *J. Biol. Chem.* **261,** 2804–2809.
36. Clari, G. & Moret, V. (1989) *Eur. J. Biochem.* **179,** 581–588.
37. Coughlin, S. R., Escobedo, J. A. & Williams, L. T. (1989) *Science* **243,** 1191–1194.
38. Endemann, G., Yonezawa, K. & Roth, R. A. (1990) *J. Biol. Chem.* **265,** 396–400.
39. Meisenhelder, J., Suh, P., Rhee, S. G. & Hunter, T. (1989) *Cell* **57,** 1109–1122.
40. Cooper, J. A., Esch, F. S., Taylor, S. S. & Hunter, T. (1984) *J. Biol. Chem.* **259,** 7835–7841.
41. Gould, K. L., Bretscher, A., Esch, F. S. & Hunter, T. (1989) *EMBO J.* **8,** 4133–4142.
42. Rees, D. J. G., Ades, S. E., Singer, S. J. & Hynes, R. O. (1990) *Nature (London)* **347,** 685–689.
43. Bretscher, A. (1989) *J. Cell Biol.* **108,** 921–930.