Fatty acid-binding protein from rat heart is phosphorylated on Tyr[19] in response to insulin stimulation

Søren U. Nielsen and Friedrich Spener[1]

Department of Biochemistry, University of Münster, Wilhelm-Klemm-Str. 2, D-4400 Münster, Germany

Abstract The cytosolic fatty acid-binding protein from rat heart (H-FABP, M_r 15 000) as well as FABP from mouse adipocytes (A-FABP, 62% homologous with H-FABP) contain a recognition sequence for protein tyrosine kinases, Asn-Phe-Asp-Asp-Tyr[19]. A-FABP has been shown by others to be partly phosphorylated on Tyr[19], thus encouraging experiments designed to search for phosphotyrosine in H-FABP. For this purpose isolated cardiac myocytes were incubated with [^{32}P]orthophosphate and analyzed by two-dimensional gel electrophoresis. A 15 kDa phosphoprotein present in the cytosolic protein fraction was specifically precipitated by a polyclonal antibody against rat heart FABP. Characterization of the phosphorylated FABP was facilitated by the development of an immunoaffinity purification procedure capable of isolating more than 200 μg FABP from four rat hearts in one step. Phosphoamino acid analysis and radiosequencing of the major tryptic phosphopeptide from immunopurified FABP revealed Tyr[19] as the phosphorylated amino acid. Stimulation of cardiac myocytes with insulin in the presence of tyrosine phosphatase inhibitors led to a several-fold increase in the amount of phosphorylated FABP compared with a nearly undetectable level found without insulin stimulation, indicating that FABP may be a substrate for the insulin receptor tyrosine kinase. Phosphorylated FABP constitutes only a minor fraction compared to the large pool of FABP in the cardiac myocyte, thus obscuring the significance of this modification. However, as the phosphorylated Tyr[19] residue is positioned within a helix-turn-helix-related domain of FABP, this modification may modulate a hitherto unknown DNA binding activity of FABP. A hypothesis is discussed in which phosphorylated FABP serves as a signalling molecule in the insulin signal transduction cascade.—**Nielsen, S. U., and F. Spener.** Fatty acid-binding protein from rat heart is phosphorylated on Tyr[19] in response to insulin stimulation. *J. Lipid Res.* 1993. **34:** 1355–1366.

Supplementary key words cardiac myocytes • insulin receptor • signal transduction • transcription factor • 2-D electrophoresis • immunoaffinity purification

The fatty acid-binding protein (FABP) from heart belongs to the group of low molecular weight intracellular proteins that bind lipophilic molecules with high affinity (reviewed in references 1 and 2). In cells with an active lipid metabolism such as cardiac myocytes, parenchymal cells of the liver, lactogenic mammary gland epithelial cells, and adipocytes, FABPs constitute 2–4% of cytosolic proteins. Interestingly, several members of this protein family contain a putative recognition sequence for protein tyrosine kinases: Asn-Phe-Asp/Glu-Asp/Glu-Tyr[19]. According to Pearson and Kemp (3) substrates for protein tyrosine kinases often contain one or more acidic amino acids just N-terminal to the phosphorylated tyrosine, and the FABP segment conforms to this motif. So far, however, phosphotyrosine has been found only in FABP from mouse adipocytes (4). This protein is also known as adipocyte lipid-binding protein (ALBP) and as the 422 or aP2 protein (4). Phosphorylation of adipocyte FABP in situ using 3T3-L1 cells as well as in vitro using purified insulin receptor (5–7) was significantly stimulated in the presence of insulin, indicating that this protein is a physiological target for the insulin receptor protein tyrosine kinase. FABP from rat, bovine, and human heart (8–10), as well as fatty acid-binding proteins isolated from lactating bovine (11) and rat mammary gland (12), contain an identical sequence around Tyr[19], suggesting the presence of phosphotyrosine also in these proteins.

The objective of the present work was to investigate whether FABP from heart can be phosphorylated on Tyr[19] in situ in response to insulin stimulation. As a cell model, the cardiac myocyte was chosen for the following reasons: *i)* this cell type contains high levels of FABP (13); *ii)* it expresses about 125,000 insulin receptors per cell (14); and *iii)* it can be obtained as a homogeneous cell population with more than 5 × 10^6 cells by Langendorff perfusion of

Abbreviations: ALBP, adipocyte lipid-binding protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2-DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FABP, fatty acid-binding protein; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; HTH, helix-turn-helix; IEF, isoelectric focusing; KRB, Krebs-Ringer buffer; PBS, 10 mM phosphate buffer, pH 7.4, with 154 mM NaCl; PMSF, phenylmethyl sulfonylfluoride; PVDF, polyvinyl difluoride; SDS, sodium dodecyl sulfate.

[1]To whom correspondence should be addressed.

one rat heart (15). For the purpose of the present study we purified FABP from rat heart and produced affinity-purified antibodies against this protein.

In our previous study, two-dimensional gel electrophoresis was applied to resolve cytosolic proteins as well as purified FABP from human heart (16, 17). Here we report for the first time the identification of heart FABP in 2-D gels of cellular proteins from isolated rat cardiac myocytes. Further, 2-DE was used to analyze phosphoproteins from [^{32}P]orthophosphate-labeled cardiac myocytes, which facilitated the successful identification of a phosphorylated isoform of cardiac FABP in this cell type. Using immunoaffinity purification, a mixture of phosphorylated and unmodified FABP was isolated and the phosphorylated isoform was characterized. We then addressed the question whether this phosphorylation is part of an intracellular response to the stimulation of myocytes by insulin.

MATERIALS AND METHODS

Affinity-purified antibodies against rat heart FABP

Using the procedure developed by Unterberg et al. (18) for the isolation of human heart FABP, about 12 mg FABP could be isolated from 140 rat hearts. Two rabbits were immunized to produce polyclonal antiserum against rat heart FABP. Affinity-purified antibodies were prepared by passing this serum through a column containing 5 mg of rat heart FABP bound to CH Sepharose 4B (Pharmacia LKB) and bound antibodies were eluted with a citric acid buffer, pH 2.8 (19).

Langendorff perfusion

Hearts from 12- to 20-week-old female Wistar rats were perfused through the aorta essentially as described by Piper et al. (15, 20). In brief, an initial 5-min perfusion with a calcium-free Krebs-Ringer bicarbonate buffer, consisting of 115 mM NaCl, 2.6 mM KCl, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 10 mM HEPES, 5 mM glucose, 20 mM taurine, continuously gassed with 95% O_2, 5% CO_2, was followed by a 30-min recirculating perfusion using KRB buffer containing 30 μM Ca^{2+} and 0.1% collagenase (Serva). The softened heart was minced with scissors and suspended with gentle agitation in KRB buffer containing 30 μM Ca^{2+} and 0.1% collagenase for another 15 min. Isolated myocytes were then obtained by filtering the suspension through a 200-μm nylon filter followed by centrifugation at 25 g to form a pellet. Four hearts were perfused simultaneously to obtain about 25 $\times$ 10^6 myocytes, at least 80% of which were rod-shaped and responded to electrical stimulation (40 V/cm, 0.5 ms) with a contraction.

^{32}P-labeling of myocytes

Isolated myocytes from four hearts were resuspended in 15 ml of labeling medium with the following composition: phosphate-free MKR buffer with 0.1 mM Ca^{2+}, essential amino acids (50 $\times$, Serva), vitamins (100 $\times$, Serva), and 1 mM orthovanadate as tyrosine phosphatase inhibitor (5). Cells were incubated at 37°C with gentle agitation for 15 min to allow cells to recover from the isolation procedure (21). Then Ca^{2+} and carrier-free [^{32}P]orthophosphate (300 MBq/nmol, ICN-Flow) were added to final concentrations of 1.0 mM and 0.3 mCi/ml, respectively, and myocytes were labeled in suspension as described by Onorato and Rudolph (22). The tyrosine phosphatase inhibitor phenylarsine oxide (5) was added after 90 min (35 μM) and insulin after 105 min (1 μM, 100 nM, or 10 nM). In control experiments addition of insulin was omitted. At the end of the 2-h labeling period, myocytes were separated from medium by centrifugation and suspended in ice-cold homogenization buffer (10 mM phosphate, 35 μM phenylarsine oxide, 1 mM VO_4^{3-}, 1 mM PMSF, 3 mM EDTA, pH 7.4). The cells were lysed by sonication for 3 $\times$ 10 sec (Branson B12 sonifier). The suspension was centrifuged at 100,000 g for 30 min and the supernatant was used for 2-D gel analysis as well as for purification of ^{32}P-labeled FABP as described in the following sections.

2-DE and Western blotting

Isoelectric focusing under denaturing conditions was performed as described by Bravo (23). IEF acrylamide gels (4% T, 5% C) containing 1% CHAPS, 1.6% Servalyte 5-7 carrier ampholytes (Serva), 0.4% Ampholines 3.5-10 carrier ampholytes (LKB), and 0.4% Biolyte 4-6 carrier ampholytes (Bio-Rad) were made in 175 $\times$ 2.1 mm i.d. (see Fig. 2) or 75 $\times$ 1.5 mm i.d. (see Figs. 3, 4, and 5) borosilicate glass tubes. With these IEF gels high resolving power between pH 4 and 7 was achieved. Second dimension gels were produced with running gel composition 18% T, 0.5% C, 375 mM Tris-HCl, 0.1% SDS, pH 8.8, and stacking gel composition 5% T, 5% C, 120 mM Tris-HCl, 0.1% SDS, pH 6.8. Western blots were made on PVDF membranes (Millipore) using a semi-dry procedure (24).

Autoradiography and staining procedures

Silver staining of 2-D gels was made according to Tunón and Johansson (25). Proteins blotted onto PVDF membranes were visualized either by colloidal gold staining (26) or by immunostaining using affinity-purified antibodies against rat heart FABP and protein A-horseradish peroxidase (Bio-Rad) as previously described (17). Autoradiography of PVDF membranes and cellulose thin-layer sheets was performed using Dupont Cronex inten-

sifying screens and Wicor X-ray films (Cea, Sweden). The exposure time was usually 4–6 days at −70°C. Autoradiography of PVDF membranes was done prior to visualization of proteins by colloidal gold or immunostaining to avoid quenching effects.

Immunoprecipitation of FABP

Affinity-purified antibodies against rat heart FABP were coupled to protein A-Sepharose CL-4B (Sigma) with dimethylpimelimidate according to Schneider et al. (27). Usually 8 mg antibodies were coupled per ml of gel matrix. Immunoprecipitation was carried out batchwise by incubating 100 μl of gel matrix with an aliquot of the 100,000 *g* supernatant from ^{32}P-labeled heart myocytes, diluted 1:1 in PBS with 2% Triton X-100. The gel matrix was washed with PBS containing 0.2% Triton X-100 and bound FABP was released by electroelution (28).

Immunopurification of FABP

FABP was purified from ^{32}P-labeled myocytes by a one-step chromatographic procedure from the 100,000 *g* supernatant. Affinity-purified antibodies were covalently coupled at 8 mg/ml to 3 ml of protein A-Sepharose CL-4B, extensively washed with PBS, and the gel matrix was packed into a C10/10 column (Pharmacia LKB) using the same buffer. The 100,000 *g* supernatant was diluted 1:1 with PBS containing 2% Triton X-100 and applied to the immunoaffinity column at a flow rate of 4 ml/h. After extensive washing with PBS containing 0.2% Triton X-100, bound FABP was eluted with 15 ml of 10 mM citrate, 20 mM NaH_2PO_4, pH 2.8. The column was equilibrated with PBS containing 0.02% NaN_3 and reused several times.

Tryptic cleavage and HPLC

Immunoaffinity-purified FABP was precipitated with 10% trichloroacetic acid and resuspended in water using a Branson B12 sonifier equipped with a microtip. After 12 min of sonication a fine suspension of denatured protein was obtained. Ammonium carbonate (pH 8.3) and TPCK-treated trypsin (Sigma) were added to final concentrations of 100 mM and 2% (w/w), respectively. After 1 h incubation at 37°C, trypsin was added to a final concentration of 4% and cleavage continued for another 3 h. Tryptic peptides were separated on a Hypersil ODS 4.6 × 125 mm column (Knauer). The flow rate was 1 ml/min and a linear gradient up to 35% buffer B (0.1% (v/v) trifluoroacetic acid in acetonitrile) in buffer A (0.1% (v/v) trifluoroacetic acid in water, pH 2) within 50 min and to 70% within a further 30 min was applied. Peptides were detected at 214 and 280 nm (Pharmacia LKB VWM 2141) and ^{32}P-radioactivity was evaluated with a HPLC radioactivity monitor (Berthold LB 507 A).

Peptide hydrolysis and 2-D thin-layer electrophoresis

Tryptic peptides containing ^{32}P-radioactivity were collected, lyophilized, and hydrolyzed in 6 M constant boiling HCl (Sigma) at 110°C for 30 min. Phosphoamino acids were identified by 2-D thin-layer electrophoresis using cellulose thin-layer chromatography plates (0.1 mm, Merck) as described by Cooper, Sefton, and Hunter (29).

Radiosequencing

Radiosequencing of the ^{32}P-labeled peptide was performed with a 477A pulsed liquid-phase sequenator (Applied Biosystems) at the Protein Analysis Facility, Department of Physiological Chemistry, University of Münster. After each Edman degradation cycle the eluate was collected and released radioactivity was determined by Cerenkov counting.

RESULTS

2-DE of proteins from rat heart myocytes

Langendorff perfusion of rat heart with collagenase (15, 20) produced a cell population of virtually pure myocytes, about 80% of which appeared rod-shaped in the microscope (**Fig. 1**). During perfusion, serum proteins are removed from the heart and accordingly the proteins separated by 2-DE of protein fractions from the cardiac myocytes (**Fig. 2**) all belong to this cell type. A few of the resolved protein spots could be allocated to abundant filament proteins and major cardiac muscle cytosolic proteins on the basis of molecular weight and apparent isoelectric point. These identifications were supported by comparison with published 2-DE patterns of proteins from human skeletal and cardiac muscle (30–32), as well as 2-DE patterns of protein fractions from rat cardiac myocytes (33). By this procedure the contractile proteins actin (44 kDa), tropomyosin (34 kDa), myosin light chain 1 (26 kDa) and 2 (20 kDa), as well as the cytosolic proteins calmodulin (20 kDa) and myoglobin (17 kDa) were localized and are marked in Figs. 2A and 2B. The position of FABP was identified by Western blotting and immunostaining using affinity purified antibodies (Figs. 2C and 2D). This identification revealed FABP as a major protein among cytosolic proteins from rat myocytes, in agreement with published data on the FABP level in rat cardiomyocytes (13). With an enzyme-linked immunoassay FABP was found to constitute 5% (± 2%, SD of four measurements) of the soluble protein fraction from rat heart myocytes (R. Rump and F. Spener, unpublished results). The immunoblotting procedure also showed the presence of minor isoforms of FABP with acidic pI values (Figs. 2C and 2D). It was anticipated that a phosphorylated isoform of FABP would migrate with a more acidic pI due to the

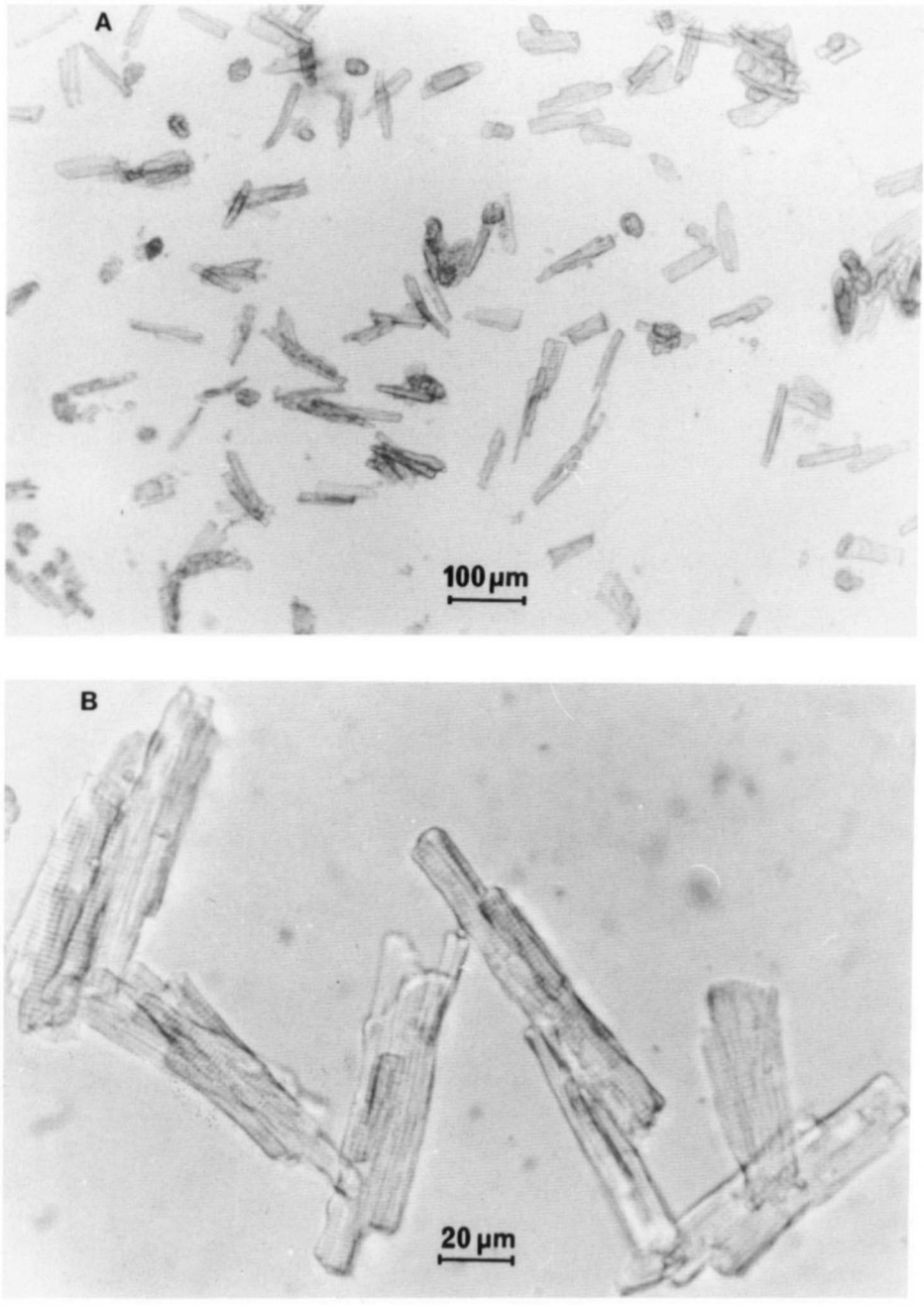

Fig. 1. Photomicrograph of isolated myocardial cells. (A), phase contrast microscopy (× 100); (B), phase contrast microscopy (× 400). The rod-shaped cells show the cross-striations typical of ventricular myocardial cells and respond to electrical stimulation with a contraction.

negatively charged phosphate group. Therefore, experiments with ^{32}P-labeled myocytes were performed to elucidate whether the presence of minor acidic isoforms of FABP was due to modification of FABP by phosphorylation.

Immunoprecipitation of ^{32}P-labeled FABP

The conditions developed by Hresko et al. (4) and Bernier, Laird, and Lane (5) to achieve maximal incorporation of [^{32}P]orthophosphate in FABP from adipocytes were used with cardiac myocytes to facilitate identification of a phosphorylated isoform of FABP. Myocytes were labeled with [^{32}P]orthophosphate in the presence of orthovanadate and phenylarsine oxide prior to stimulation with insulin. From an aliquot of the 100,000 *g* supernatant, FABP was immunoprecipitated with 0.8 mg of affinity-purified antibodies against rat heart FABP (**Fig. 3A**). The precipitate

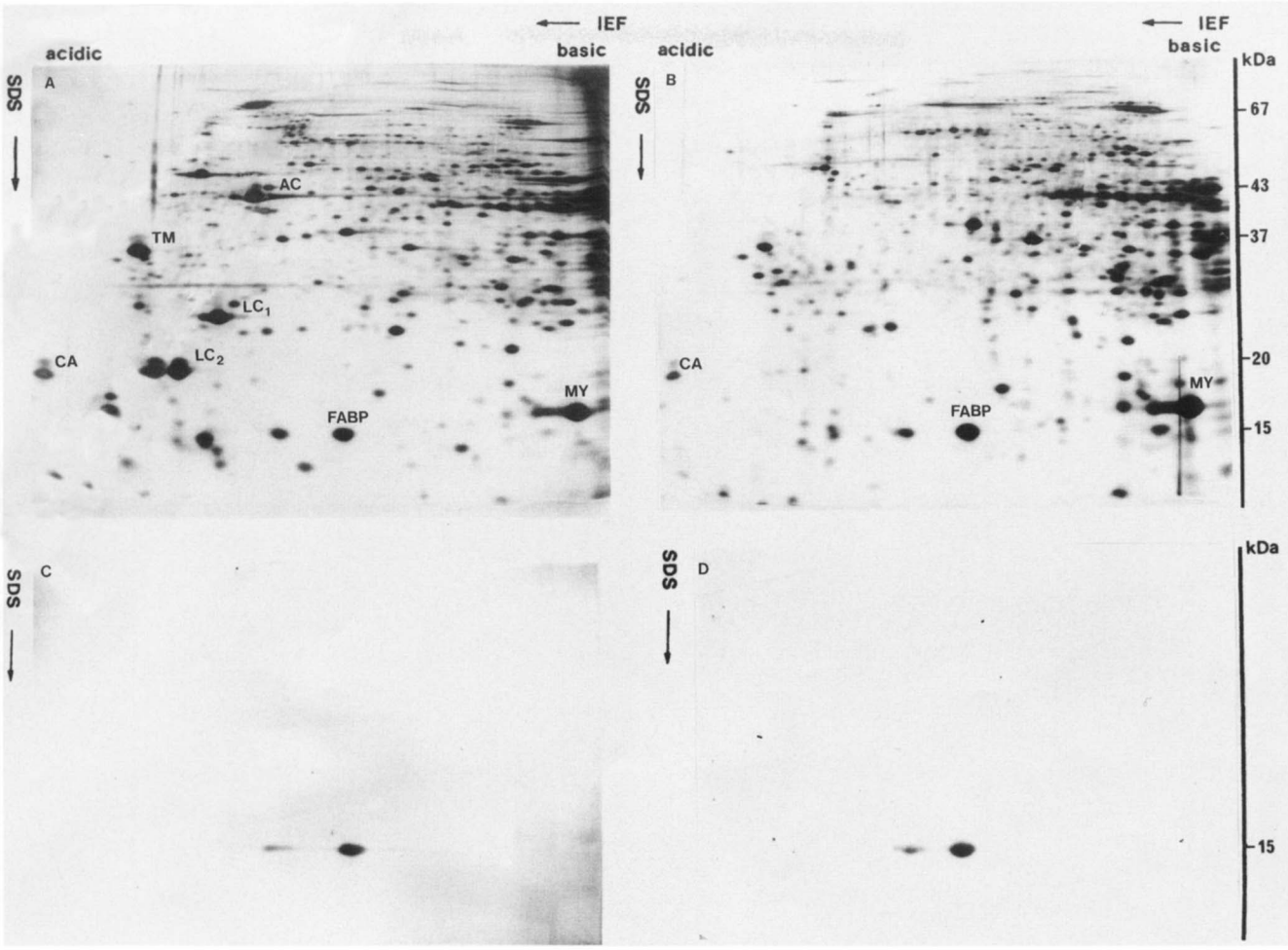

Fig. 2. 2-DE of total cellular proteins (A, C) or proteins from the 100,000 *g* supernatant (B, D) from isolated rat heart myocytes. Major filament proteins as well as some cytosolic proteins are marked with arrows: AC, Actin; TM, tropomyosin; LC_1 and LC_2, myosin light chains 1 and 2, respectively; MY, myoglobin; CA, calmodulin. Loading about 150 μg, silver stain (A, B). Detection of FABP by Western blotting (C, D). Total cellular proteins (C) or proteins from the 100,000 *g* supernatant (D) from cardiac myocytes were incubated with affinity purified antibodies against rat heart FABP.

contained the major FABP isoform with a pI around 6.0 as well as minor isoforms with more acidic pIs. Autoradiography (Fig. 3C) showed coprecipitation of a 15-kDa phosphoprotein with a pI close to those of the minor acidic FABP isoforms. According to Boyle, van der Geer, and Hunter (34) the expected charge of an P-Ser, P-Thr, and P-Tyr residue at pH 5–6 is in the range of −1 to −1.2. Therefore, the more acidic pI of the phosphorylated FABP can be attributed to an extra negative charge on the phosphorylated amino acid. When isoelectric focusing was performed under native conditions, without urea and DTT, rat heart FABP showed a major isoform with a pI about 4.8 as well as minor isoforms with more acidic pIs (data not shown). A shift in pI upon denaturation has also been observed with human heart FABP (16, 17).

For a competition experiment, a large excess (1 mg) of unlabeled, purified rat heart FABP was added to the cytosolic protein fraction from ^{32}P-labeled myocytes prior to immunoprecipitation. Due to a limited binding capacity of the antibody-Sepharose matrix, the amount of FABP precipitated was unaffected by this addition (compare Figs. 3A and 3B). However, the corresponding autoradiogram from a 2-D gel of immunoprecipitated FABP demonstrated a significant reduction in the intensity of ^{32}P-labeled FABP (Fig. 3D), confirming that this phosphoprotein corresponded to phosphorylated FABP. Thus, the isoform of rat heart FABP with the most acidic pI is the phosphorylated isoform. This isoform, however, constitutes only a minor fraction of the total pool of FABP in the cardiac myocyte.

Phosphorylation of FABP is stimulated by insulin

To evaluate the effect of insulin on phosphorylation of FABP, cardiac myocytes were labeled with [^{32}P]orthophosphate in the presence of tyrosine phosphatase inhibi-

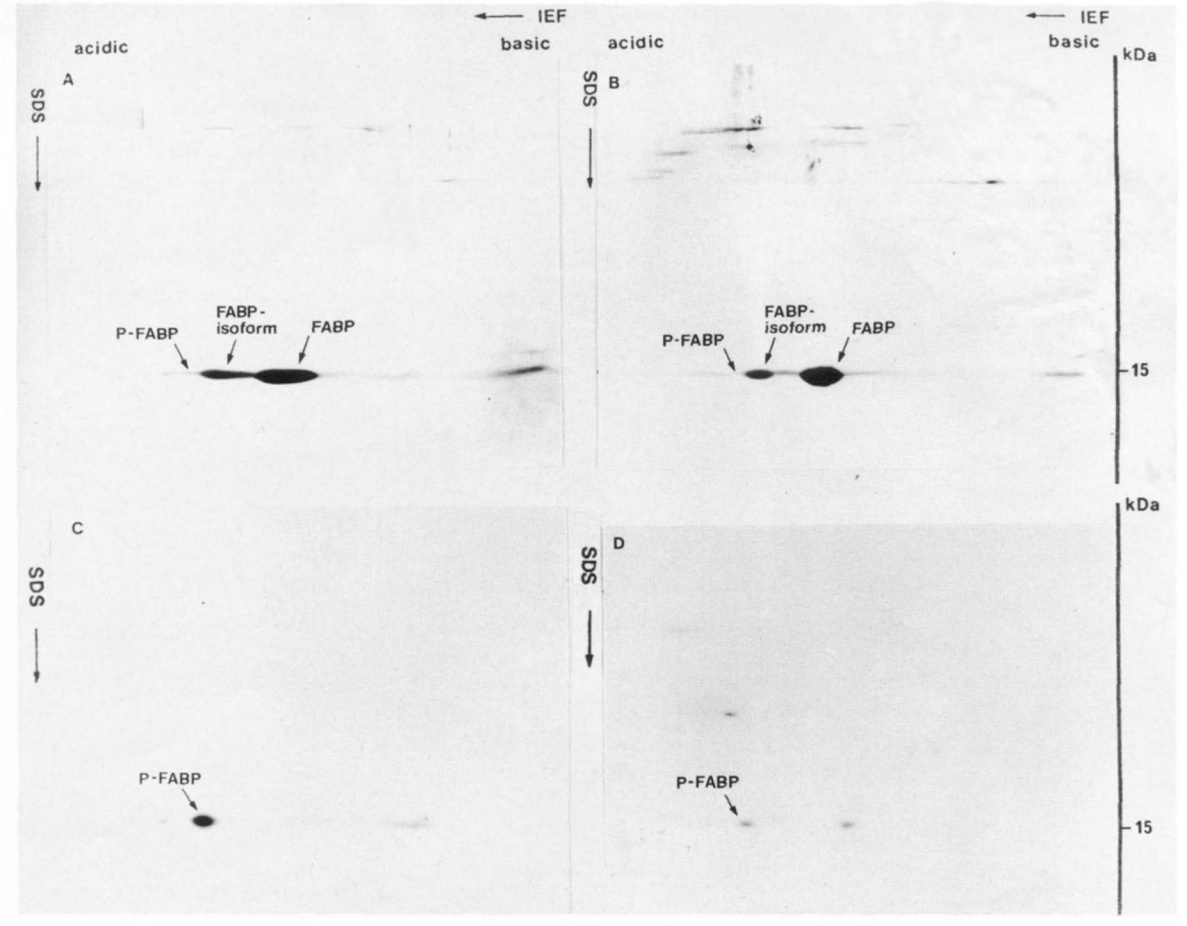

Fig. 3. Immunoprecipitation of FABP from ^{32}P-labeled rat heart myocytes without (A, C) and with competition from unlabeled FABP (B, D). An aliquot of the 100,000 *g* supernatant from ^{32}P-labeled myocytes was incubated with 100 μl of protein A-Sepharose CL-4B covalently linked with 0.8 mg of affinity-purified antibodies against rat heart FABP. The Western blot from 2-DE of the precipitate was stained with colloidal gold (A) and subjected to autoradiography (C). (B, D), immunoprecipitation as described above, apart from addition of 1 mg unlabeled FABP to the 100,000 *g* supernatant aliquot prior to precipitation. The Western blot of the precipitate was stained with colloidal gold (B) and subjected to autoradiography (D). The positions of FABP and the putative Asn/Asp-exchange product of FABP (FABP-isoform) as well as the phosphorylated FABP (P-FABP) are marked.

tors, either with or without insulin stimulation. The 100,000 *g* supernatant was produced and analyzed by 2-DE with subsequent Western blotting and autoradiography. With insulin stimulation three isoforms of FABP could be visualized by immunostaining (**Fig. 4A**). In this experiment the minor acidic isoform of FABP was clearly resolved in two spots, the most acidic of which corresponded to the phosphorylated isoform (compare Figs. 4A and 4B). The other acidic isoform may be due to an exchange of Asn by Asp in FABP adding one negative charge to the protein. Modification of Asn^{98} to Asp^{98} was found to cause the occurrence of the two isoelectric isoforms observed in bovine heart FABP (35). In the absence of insulin stimulation, a pronounced decrease in the intensity of the spot corresponding to phosphorylated FABP was observed (Fig. 4C). The intensity of most other phosphoproteins, however, was unaffected by insulin stimulation (compare Figs. 4B and 4C). A group of 44-kDa phosphoproteins as well as a 21-kDa phosphoprotein showed a high degree of ^{32}P incorporation in the absence of insulin, but became almost completely dephosphorylated with insulin stimulation (compare Figs. 4C and 4B). The identity of these proteins has not been determined.

The requirement for tyrosine phosphatase inhibitors for the detection of phosphorylated FABP was evaluated. For this purpose myocytes were labeled with [^{32}P]orthophosphate with and without orthovanadate and phenylarsine oxide, followed by 15 min of stimulation with 10 nM insulin. Phosphorylation of FABP was detected at this near physiological insulin concentration as well (**Fig. 5A**). Omission of tyrosine phosphatase inhibitors led to a decreased, but still detectable, level of phosphorylated

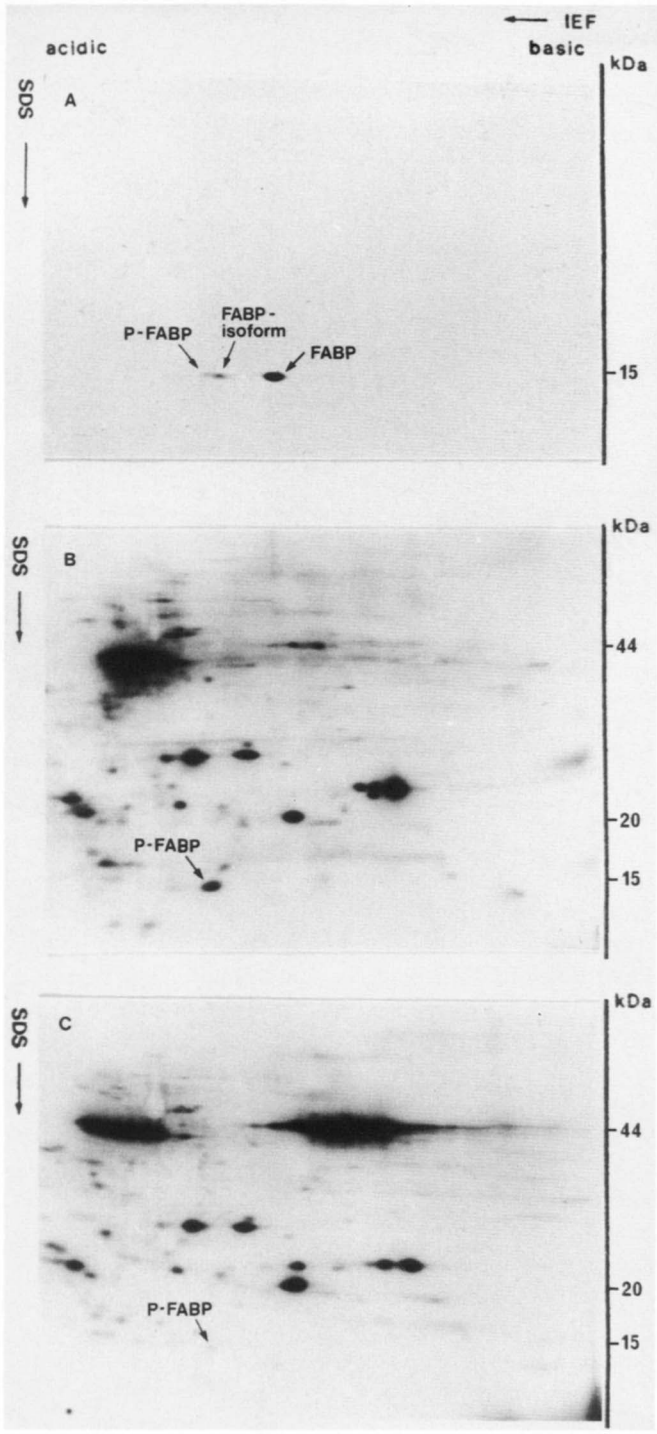

Fig. 4. Evaluation of effects of insulin stimulation on the phosphoprotein pattern from rat heart myocytes. (A), Western blot of proteins from the 100,000 *g* supernatant of cardiac myocytes incubated with [^{32}P]orthophosphate in the presence of orthovanadate and phenylarsine oxide and with insulin stimulation. Immunostaining with affinity-purified antibodies against rat heart FABP. (B), Autoradiogram of the Western blot shown in (A). (C), Autoradiogram of a Western blot of proteins from the 100,000 *g* supernatant from cardiac myocytes incubated with [^{32}P]orthophosphate in the presence of orthovanadate and phenylarsine oxide but without insulin stimulation. The positions of FABP and the putative Asn/Asp exchange product of FABP (FABP-isoform) as well as phosphorylated FABP (P-FABP) are marked.

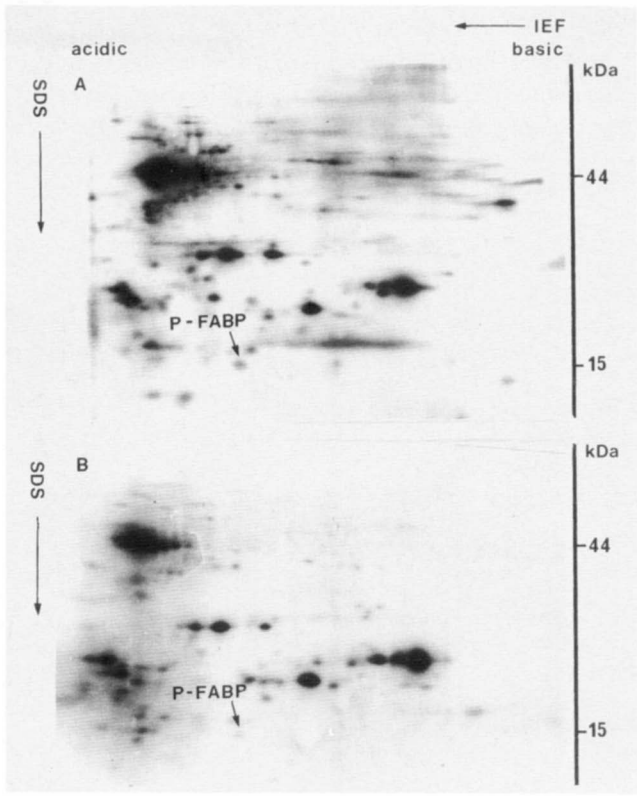

Fig. 5. Evaluation of the requirement for tyrosine phosphatase inhibitors for the detection of insulin-stimulated phosphorylation of FABP. Cardiac myocytes were labeled with [^{32}P]orthophosphate with (A) and without (B) the presence of orthovanadate and phenylarsine oxide. After 105 min of labeling, cells were stimulated with 10 nM insulin for another 15 min followed by homogenization and 100,000 *g* centrifugation. Autoradiograms from 2-D gels of the 100,000 *g* supernatants are shown. The position of phosphorylated FABP (P-FABP) is indicated.

FABP (Fig. 5B). Similar results were obtained with 100 nM of insulin, whereas epidermal growth factor (20 ng/ml) failed to stimulate FABP phosphorylation (data not shown).

Immunopurification of ^{32}P-labeled FABP

To produce ^{32}P-labeled FABP in sufficient amounts to allow identification of the phosphorylated amino acid by phosphoamino acid analysis of tryptic peptides, a chromatographic immunoaffinity purification procedure was adapted (27). A column with protein A-Sepharose containing 24 mg of affinity-purified antibodies against rat heart FABP efficiently and specifically extracted FABP from the 100,000 *g* supernatant of ^{32}P-labeled heart myocytes. Bound FABP was eluted with citrate buffer, pH 2.8, and precipitated by addition of trichloroacetic acid to 10% final concentration. The purity of this preparation as evaluated by 2-DE was the same as that obtained by the batchwise immunoprecipitation (Fig. 3A). About 0.5 mg FABP could be purified from myocytes of eight hearts.

Identification of phosphotyrosine in FABP

A tryptic digest of immunopurified FABP from ^{32}P-labeled myocytes was separated by reversed-phase HPLC (**Fig. 6**) and absorbance was followed at 214 and 280 nm

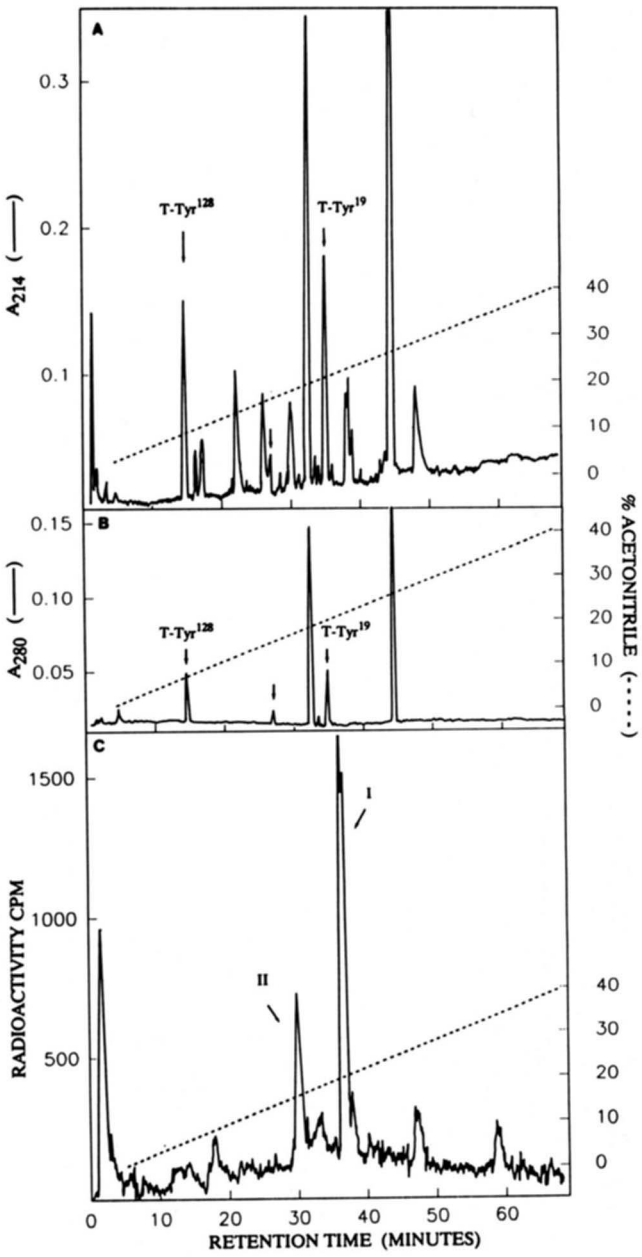

Fig. 6. Separation of tryptic peptides from ^{32}P-labeled FABP by reversed-phase HPLC. FABP was isolated by immunoaffinity purification from ^{32}P-labeled cardiac myocytes treated with orthovanadate, phenylarsine oxide, and insulin. Peptides were eluted from the RP-18 column with an acetonitrile gradient (----) and monitored at 214 nm (A) and 280 nm (B). Radioactivity was determined by Cerenkov counting (C). Tryptic peptides containing Tyr^{19} or Tyr^{128} are marked T-Tyr^{19} and T-Tyr^{128}, respectively. Two phosphopeptides are labeled I and II, respectively. The arrow in Fig. 6A and 6B indicates the peptide that is the putative nonspecific cleavage product of the T-Tyr^{19} peptide, as described in the legend to Fig. 9.

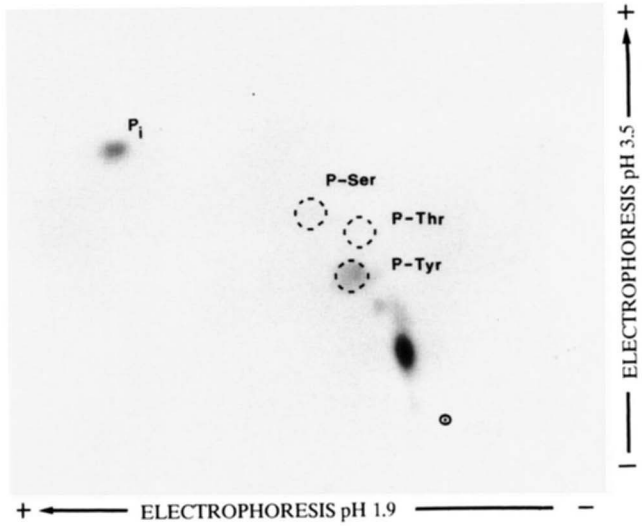

Fig. 7. Phosphoamino acid analysis of ^{32}P-labeled FABP. FABP was isolated by immunoaffinity purification from ^{32}P-labeled cardiac myocytes and subjected to trypsin digestion. Phosphopeptide I from HPLC analysis of tryptic peptides (see Fig. 6) was hydrolyzed in 6 N HCl and analyzed by 2-D thin-layer electrophoresis. The autoradiogram shows phosphotyrosine as the only labeled phosphoamino acid. The point of application (O), released inorganic ^{32}P (P_i), as well as the position of standard phosphoamino acids (stippled circles) are marked. A major spot above the point of application corresponds to incompletely hydrolyzed phosphopeptides.

to facilitate identification of peptides containing tyrosine or tryptophan residues. By comparison with published tryptic peptide maps of rat heart FABP (8) and bovine heart FABP (35) the peptides containing the two tyrosines in rat heart FABP (T-Tyr^{19} and T-Tyr^{128} in Figs. 6A and 6B) were identified. Monitoring of radioactivity by Cerenkov counting showed two major peaks containing about 1600 cpm and 700 cpm, respectively (labeled phosphopeptide I and II in Fig. 6C). Phosphopeptide I eluted close to the Tyr^{19}-containing peptide, whereas phosphopeptide II eluted about 7 min earlier. Phosphoamino acid analysis of peak I by 2-D thin-layer electrophoresis showed the presence of phosphotyrosine (**Fig. 7**). A preparation of HPLC-purified phosphopeptide I containing 1200 cpm was analyzed by automated Edman degradation. The minute amounts of peptide excluded determination of amino acids and therefore the complete eluate was collected after each degradation cycle and analyzed by Cerenkov counting. Radioactivity was released only in the fifth cycle (**Fig. 8A**) in agreement with the location of tyrosine in position 5 of the tryptic peptide containing Tyr^{19} (Fig. 8B), thus confirming that this residue is phosphorylated.

The possibility was evaluated that phosphopeptide II, which also contained phosphotyrosine as judged by thin-layer electrophoresis (data not shown), was a nonspecific cleavage product of phosphopeptide I. For this purpose peptide T-Tyr^{19} was redigested with chymotrypsin and

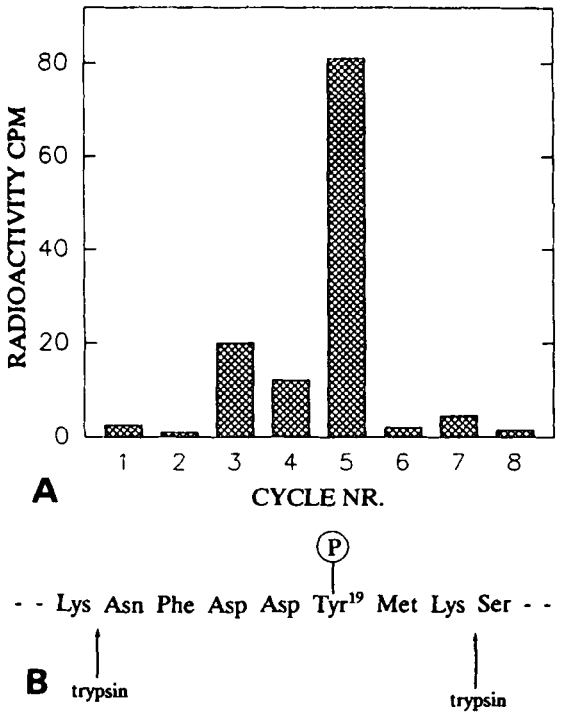

Fig. 8. Identification of the phosphorylated amino acid in FABP by radiosequencing. (A), Phosphopeptide I (see Fig. 6) was subjected to automated Edman degradation and released radioactivity was determined after each cycle by Cerenkov counting. (B), Amino acid sequence surrounding Tyr^{19} in rat heart FABP. In the tryptic peptide Tyr^{19} occurs at position 5 in agreement with the observed release of ^{32}P-radioactivity in cycle 5 of the Edman degradation procedure (Fig. 8A).

subjected to rechromatography. A new peak eluted at 27 min, about 7 min prior to the uncleaved T-Tyr^{19} peptide, possibly due to cleavage C-terminally to Phe^{16} or Tyr^{19} (labeled TC-Tyr^{19} in **Figs. 9A and 9B**). Presumably as a result of a chymotryptical contamination or a tryptical side-activity, a tyrosine-containing peptide with the same retention time was observed on the tryptic peptide map of FABP (arrow in Figs. 6A and 6B). Thus, phosphopeptide II is likely to be the phosphorylated form of this nonspecific cleavage product, also phosphorylated on Tyr^{19}.

As evident from Figs. 6 and 9, phosphopeptides I and II elute about 3 min later from the C_{18} column than their unphosphorylated counterparts. This behavior was somewhat unexpected as phosphorylation adds one negative charge to the peptide at a pH of 2 (34), thus making it less hydrophobic. Cohen, Gibson, and Holmes (36) found that phosphopeptides containing P-Ser usually eluted prior to the unphosphorylated peptide on a C_{18} column, using eluents similar to those described in the present study. However, exceptions were found in which phosphorylated and unphosphorylated peptides eluted together (36). The ionization state of the phosphorylated Tyr^{19} residue at pH 2 may be affected by the water/acetonitrile mixture in a way that somehow reinforces its interactions with the C_{18} material, thus causing a delayed elution of the peptide containing P-Tyr^{19} from the column.

DISCUSSION

In the present study we have demonstrated that the fatty acid-binding protein from isolated rat heart myocytes is partially phosphorylated on Tyr^{19}, apparently as a physiological substrate for the insulin receptor tyrosine kinase. This is the second observation of phosphotyrosine among FABPs; the first identification was made by Hresko et al. (4) with mouse adipocyte FABP, which shows 62% homology to heart FABP. The tyrosine kinase recognition sequence surrounding Tyr^{19} is identical in adipocyte and heart FABP and is additionally found in the myelin P2 protein as well as in the cellular retinol binding protein, suggesting that other members of the FABP family may contain this modification.

Until now only a few proteins, including ALBP, pp120, and pp185, have been identified as physiological substrates for the insulin receptor tyrosine kinase (37). This

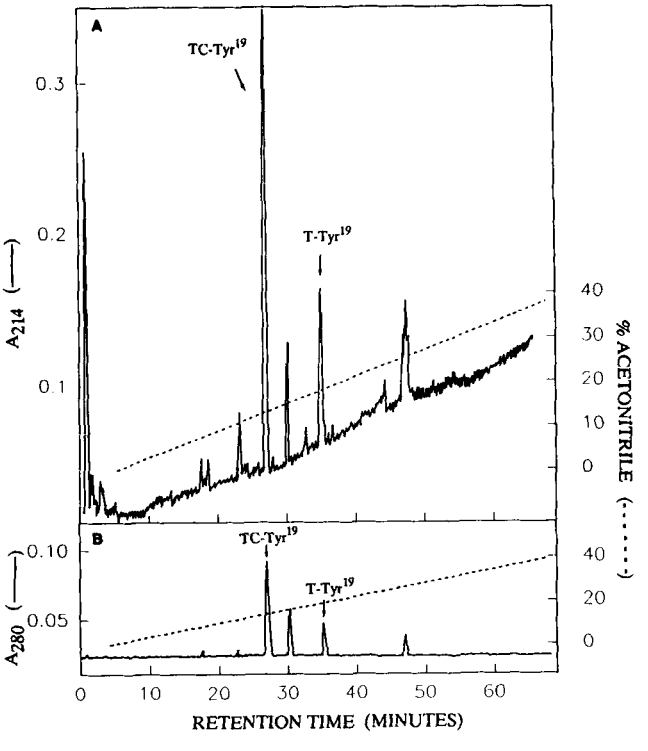

Fig. 9. Rechromatography of peptide T-Tyr^{19} after digestion with chymotrypsin. Peptides were eluted from the reversed-phase RP-18 column with an acetonitrile gradient and absorption was followed at 214 nm (A) and 280 nm (B). A new peptide is observed prior to the uncleaved T-Tyr^{19} peptide. This is a cleavage product of the T-Tyr^{19}, possibly due to cleavage C-terminal to the Phe^{16} or Tyr^{19} residues in the sequence Asn-Phe-Asp-Asp-Tyr^{19}. This peptide is marked TC-Tyr^{19}. A peptide with similar retention time was observed when FABP was cleaved with trypsin (arrow in Fig. 6A and 6B).

report adds heart FABP as a candidate to this list, thus stressing that the FABPs may be important participants in the propagation of the insulin signal in insulin responsive tissues.

Hresko et al. (4) showed that accumulation of phosphorylated FABP in adipocytes required the presence of tyrosine phosphatase inhibitors and stimulation with insulin to obtain an intracellular concentration of 0.3 μM. Thus, with an intracellular pool of about 200 μM FABP in adipocytes (5), less than 0.2% of the FABP population occurs in the phosphorylated form. In the present work no quantitative evaluation of the amount of phosphorylated FABP in the cardiomyocyte was made. However, it is evident from the 2-D gels and HPLC diagrams of immunopurified ^{32}P-labeled FABP presented here (Figs. 3–6) that only a minute fraction of FABP is in the phosphorylated form in the cardiac myocyte. Insulin-stimulated phosphorylation of FABP in cardiomyocytes was detected also in the absence of orthovanadate and phenylarsine oxide, albeit with a diminished cellular concentration. This is in contrast to the observations by Hresko et al. (4) made with insulin-stimulated adipocytes, where phosphorylation of ALBP required the presence of at least one of these tyrosine phosphatase inhibitors. As reviewed by Glenney (38), tyrosine phosphorylation of proteins often occurs with very low stoichiometry, thus making the function of this modification an enigma. Possible functions for the phosphorylation of cardiac FABP or the homologous protein adipocyte FABP (ALBP) are at present only speculative. Hresko et al. (6) suggested that phosphorylation of ALBP decreases its capacity to bind and transport fatty acids, thereby leading to their accumulation in the cell. This effect is thought to mediate the antilipolytic function of insulin in adipocytes through the feedback inhibition of lipolysis by free fatty acids. A second suggestion has been made by Buelt et al. (39) based on experimental evidence which showed that phosphorylation of ALBP inhibits free exchange of ligand. In this model phosphorylation/dephosphorylation of ALBP regulates fatty acid uptake and trafficking in adipocytes. It is anticipated that ALBP with bound fatty acid becomes dephosphorylated on cellular membranes by a membrane-bound tyrosine phosphatase thereby making exchange of ligand between ALBP and the membrane possible. However, both these models are seriously hampered by the observed low stoichiometry of phosphorylation, leaving about 99.8% of the ALBP population in the unmodified form and therefore unable to contribute to the suggested cellular effects of phosphorylation.

A theory for the cellular action of phosphorylated FABP must take into account the low stoichiometry of phosphorylation, which under normal physiological conditions with insulin stimulation in the absence of tyrosine phosphatase inhibitors is far below 0.2%. In addition, it must incorporate the findings by Hresko et al. (6) and Buelt et al. (7) that FABP with bound long-chain fatty acid is a much better substrate for the insulin receptor in vitro than the apoprotein and, accordingly, the holoprotein is the likely in vivo substrate. Based on these requirements a third model for the cellular function for phosphorylated FABP is proposed, which maintains the basic assumption made in the model suggested by Hresko et al. (6), namely that phosphorylated FABP fulfills a role in the cellular propagation of the insulin signal. In our model phosphorylated FABP serves as a signalling

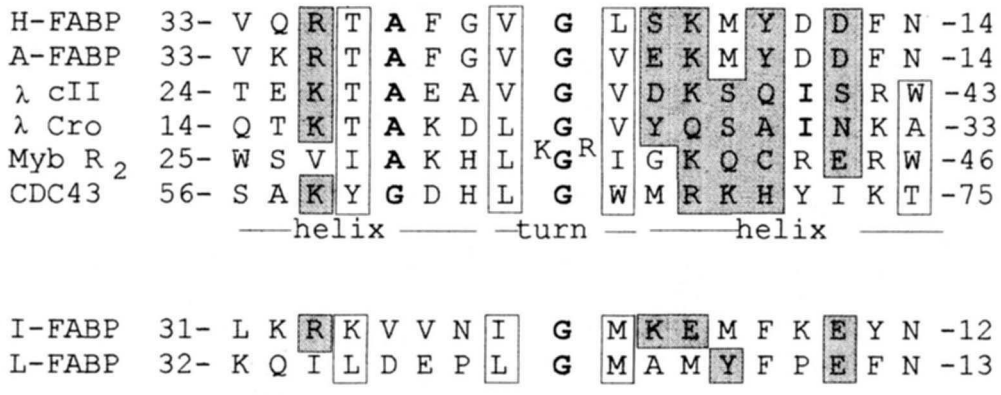

Fig. 10. Constrained alignment of a helix-turn-helix-related motif in FABP from rat heart (H-FABP) and mouse adipocytes (A-FABP) with HTH motifs from bacteriophage λ proteins (λ Cro and λ cII (43)) as well as from eucaryotic proteins (Myb R_2 (44) and CDC43 (45)). Internal sequences of FABP from rat intestine (I-FABP) and rat liver (L-FABP) (1), each containing two α-helices and a turn, are added for comparison. Positions with strongly conserved amino acids in the HTH motif are in bold face. Positions with generally or highly hydrophobic amino acids are surrounded by an unshaded box and positions with polar amino acids are surrounded by a shaded box. The sequences from the FABPs are read in reverse, e.g., from the C-terminal to the N-terminal direction, as this type of alignment results in the highest degree of homology to the HTH motif. The numbers at the beginning and end of each line are the residue numbers in the protein sequence.

molecule downstream from the activated insulin receptor tyrosine kinase, formation of which requires the presence of long-chain fatty acids in the cell. This mechanism would confer fatty acid sensitivity to the insulin signal transduction chain and secure that the insulin signal is propagated only if these metabolites are available. Such a function can be achieved even with a minute cellular concentration of phosphorylated FABP and has no impact on the participation of the major FABP population in cellular fatty acid uptake and trafficking. The model implies that holoFABP through phosphorylation on Tyr^{19} acquires the ability to interact with more distal components of the insulin signal transduction cascade, presumably via binding, as FABP is devoid of enzymatic activity.

Interestingly, the phosphorylated Tyr^{19} residue is located within a domain of FABP with two α-helices and a turn, which shows similarities to the helix-turn-helix (HTH) motif (40). By alignment of this region with sequences from the HTH domain of prokaryotic and eucaryotic proteins, considerable homology is found (**Fig. 10**). In particular, a constrained alignment in which the FABP sequence is read in reversed direction brings a high homology. Moreover, by this alignment the Tyr^{19} residue becomes positioned within the so-called recognition helix, which in prokaryotic HTH-proteins determines the DNA sequence specificity. I-FABP and L-FABP, which possess no phosphotyrosine, show less homology to the HTH motif. In our model, the presence of a HTH-related domain in cardiac and adipocyte FABP identifies DNA as one putative candidate for a cellular target of phosphorylated FABP. Interesting examples of a regulatory principle by which the activity of a latent cytoplasmic transcription factor is modulated by phosphorylation are found in the recent literature. *i)* Schindler et al. (41) identified a cytoplasmic 54 kDa protein which was shown to become phosphorylated on tyrosine after stimulation of the cells with interferon. This phosphorylation converted the protein to a transcription factor that was translocated to the nucleus to enhance expression of interferon induced genes. *ii)* Bajar, Podila, and Kolattukudy (42) described a transcription factor in a plant/fungal system that required the presence of a ligand in addition to phosphorylation to become active. In the presence of cutin monomers released from infected plant organs, a fungal protein became phosphorylated and was thereby converted to a transcription factor which induced the expression of the cutinase gene in the fungus. Upon dephosphorylation of the transcription factor or in the absence of cutin monomers, no induction took place. However, further work, especially using purified phosphorylated H-FABP, is needed to evaluate whether the phosphorylation of H-FABP renders this protein able to interact with cellular components in the insulin signal transduction cascade.

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