Abnormal Protein Tyrosine Phosphorylation in Fibroblasts from Hyperapobetalipoproteinemia Subjects*

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Mahnaz Motevalli, Pascal J. Goldschmidt-Clermont, Donna Virgil, and Peter O. Kwiterovich, Jr.¶‡

From the Lipid Research Atherosclerosis Unit, Division of Cardiology, Bernard Laboratory, Departments of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-3654

The stimulatory effects of three normal human serum basic proteins (BP), BP I (Mr 14,000, pI 9.10), BP II (Mr 27, 500, pI 8.49), and BP III (Mr 55,000, pI 8.73) on cellular triglyceride and cholesterol formation require intact protein-tyrosine kinase phosphorylation (TKP). Here we examined whether there is an abnormality in TKP in cultured fibroblasts from 11 patients with hyperapobetalipoproteinemia (hyperapoB) that manifest two acylation-stimulatory defects, decreased stimulation of triglyceride synthesis by BP I but enhanced formation of cholesterol by BP II. Soluble and insoluble proteins in Triton X-100 extracts were isolated by immunoprecipitation with a monoclonal anti-phosphotyrosine antibody (MAPA) bound to agarose beads and by ultracentrifugation, respectively, from confluent fibroblasts after incubation for 24 h in supplemented serum-free and lipid-free medium (DMEM/F12). Western blots of insoluble proteins showed that group (Gp) II (Mr 36,000–55,000) and Gp III (Mr 14,000–35,000) from hyperapoB cells, grown in DMEM/F12 medium without BP, had significantly decreased reactivity to MAPA. No significant differences in reactivity to MAPA were detected between normal and hyperapoB cells for Gp I (Mr 97–120,000). BP II, but not BP I or BP III, reversed the decreased reactivity of Gp II and Gp III to MAPA in hyperapoB cells. Sodium vanadate, an inhibitor of phosphotyrosine phosphatases, did not reverse the deficiency in TKP or the 50% deficiency in the stimulation of mass triglyceride by BP I in hyperapoB cells. Tyrosine-phosphorylated Erk-2, a mitogen-activated protein kinase, identified as one of the proteins in Gp II, was significantly decreased in hyperapoB cells. These results provide further evidence for abnormal protein TKP in hyperapoB cells and suggest a possible link between atherosclerotic changes in hyperapoB patients and growth factors upstream from mitogen-activated protein kinase.

Hyperapobetalipoproteinemia (hyperapoB) is a lipoprotein disorder that is prevalent in patients with premature coronary artery disease (1, 2). HyperapoB is characterized by an increased number of small, dense low density lipoprotein (LDL) particles, a phenotype shared with familial combined hyperlipidemia, LDL subclass pattern B, familial dyslipidemic hyper tension, and syndrome X (2, 3). Two metabolic defects have been described in hyperapoB. First, there is overproduction of apolipoprotein B and very low density lipoprotein (VLDL) particles (3, 4). Second, the clearance of postprandial triglyceriderich particles is delayed, accompanied by an abnormal removal of free fatty acids (5, 6). Incorporation of free fatty acids into triglycerides is deficient in hyperapoB adipocytes, which may lead to an increase in postprandial free fatty acids, which then flux back to the liver, leading to overproduction of apolipoprotein B and VLDL (3–7).

We (8–12) and others (13–18) have studied the role of certain human serum basic proteins that have been linked to the pathogenesis of hyperapoB. We isolated three basic proteins (BP) from normal human serum that we called BP I, BP II, and BP III, based on their electrophoretic migration (8). Their Mr and isoelectric points, respectively, were as follows: BP I, 14,000 and 9.10; BP II, 27,500 and 8.48; BP III, 55,000 and 8.73 (8). The amino acid compositions of each were distinct from each other (8). BP I appears to be a different protein from acylation-stimulatory protein, a basic protein that has similar metabolic effects; acylation-stimulatory protein has been reported to be the same protein as C3a des-Arg (Mr 8,000), a proteolytic cleavage product of C3a, the third component of complement (15). In normal cultured fibroblasts, the major effect of BP I is a marked (2–3-fold) stimulation of the mass of triglyceride (8–11). BP II and BP III have considerably less of an effect than BP I on triglyceride formation in normal cells.

The effects of BP I, BP II, and BP III on triglyceride and cholesterol metabolism in hyperapoB fibroblasts are clearly different from each other (8–12). First, there is a 50% deficiency in the stimulation of triglyceride production by BP I in hyperapoB fibroblasts. No abnormality in triglyceride metabolism was seen with BP II or BP III in hyperapoB cells (8–12). Second, BP II (but not BP I or BP III) abnormally stimulated (about 6-fold) the production of cholesterol and cholesteryl esters in hyperapoB fibroblasts. If such an effect of BP II occurs in the liver of hyperapoB patients, this may further accentuate hepatic apolipoprotein B and VLDL overproduction, leading to overproduction of LDL and increased atherosclerosis (3). There protein(s); BP, basic protein(s); Erk-2, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; DMEM/F12, supplemented serum-free, lipid-free medium; PBS, phosphate-buffered saline; TBS-T, Tris buffered saline with 0.5% Tween; TKP, protein-tyrosine kinase phosphorylation; PAGE, polyacrylamide gel electrophoresis; Gp, group; MAPK pathway, Grb2/Sos/Ras/Raf/Mek/Erk pathway.

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¶ To whom correspondence should be addressed: Lipid Research Atherosclerosis Unit, The Johns Hopkins Hospital, 600 N. Wolfe Street/CMSC 604, Baltimore, MD 21287-3654. Tel.: 410-955-3197; Fax: 410-955-1276.

The abbreviations used are: hyperapoB, hyperapobetalipoproteinemia; LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); BP, basic protein(s); Erk-2, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; DMEM/F12, supplemented serum-free, lipid-free medium; PBS, phosphate-buffered saline; TBS-T, Tris buffered saline with 0.5% Tween; TKP, protein-tyrosine kinase phosphorylation; PAGE, polyacrylamide gel electrophoresis; Gp, group; MAPK pathway, Grb2/Sos/Ras/Raf/Mek/Erk pathway.
was no abnormality in triglyceride or cholesterol metabolism in hyperapoB fibroblasts with BP III.

In cultured normal human monocyte-derived macrophages, BP III, but not BP I or BP II, significantly stimulated the formation of cell cholesteryl esters (9). This suggests the possibility that the effects of the serum basic proteins may be tissue-specific and provides further evidence for structural and functional differences between BP I, BP II, and BP III.

In normal cultured fibroblasts, the effects of BP I and BP II are time- and concentration-dependent (8–12), suggesting that a high affinity cell surface mechanism is involved. In hyperapoB cells, the defect in the response to the stimulation of triglyceride formation by BP I is also time- and concentration-dependent and appears related to a deficiency in a high affinity cell surface mechanism (19). Conversely, the abnormality in the overresponse of hyperapoB cells to BP II by enhanced cholesterol formation is concentration-dependent and saturable (7, 8) and appears due to an increased interaction of BP II with the cell surface (19).

These effects of BP I and BP II were blocked by genistein, a specific inhibitor of protein-tyrosine kinase phosphorylation (TKP) (20, 21). The stimulatory effect of BP I on the production of triglyceride was inhibited by 50% by genistein in normal cells, to a level seen in hyperapoB cells (11). In contrast, no inhibition of this effect of BP I was seen with genistein in hyperapoB cells, suggesting that the defect in the response of hyperapoB cells to the acylation-stimulatory activity of BP I may involve TKP. The abnormal stimulation by BP II of cholesterol mass and production in hyperapoB cells was also inhibited by genistein (12). These observed biochemical effects of genistein may be due to a generalized effect on TKP, which might include transmembrane tyrosine kinase receptors, membrane-associated tyrosine kinase molecules, or protein kinases at the postreceptor level (22–24).

An important component of atherosclerosis is uncontrolled cell growth in the vessel wall. Since tyrosine kinases are involved in the control of cell growth, an abnormality in the tyrosine kinase-mediated second messenger pathways may contribute to increased cell proliferation in the arterial wall and premature atherosclerosis in patients with hyperapoB.

Here we have taken a more direct immunologic approach to study the possibility that there is an abnormality in TKP in hyperapoB cells and the effect of BP I, BP II, and BP III on TKP. We also examined the immunoreactivity of tyrosine-phosphorylated extracellular signal regulatory kinase-2 (Erk-2), a mitogen-activated protein kinase (MAPK), known to be activated as a result of the effect of growth factors on cell surface transmembrane tyrosine kinase receptors (25–27).

EXPERIMENTAL PROCEDURES

Patient Population—Six hyperapoB probands (BB, GC, CH, CL, BO, and WY) from six unrelated kindreds with familial hyperapoB and six unrelated normal subjects have been characterized previously (8–12). Six first degree relatives of three of the probands were also studied here: TL, sister of BO; WB and RB, father and brother of BB; and EY, KG, and JL, brother, daughter, and grandson of WY. All but one of the relatives (JL) had hyperapoB defined as an elevated LDL apoB level (>120 mg/dl) with a normal LDL cholesterol level (8).

Human Serum Basic Proteins—BP I, BP II, and BP III were isolated from normal subjects as described previously (8–11), with the following modifications. For the initial purification step, normal human serum (25 ml) was applied to a DEAE-Affi Blue Column (2.5 cm, 32 cm). Several unretained peaks containing a mixture of basic proteins were eluted with 0.02 M phosphate, pH 8.0. Peak I was used to isolate BP II and BP III. Several retained peaks were eluted with a salt gradient (0.1–2.0 M NaCl in 0.02 M phosphate buffer, pH 8.0), and peak I (retained) was used to isolate BP I. Following isolation by preparative SDS gel electrophoresis as described (8–11), each protein was homogeneous by reverse phase high pressure liquid chromatography. The presence of C3a des-Arg (15) in peak I (unretained) and peak I (retained) and in purified BP I, BP II, and BP III was assessed using a commercial assay RIA kit for C3a des-Arg (Amersham Corp.). The percentage of the total protein that was immunoochemically reactive to anti-C3a des-Arg was as follows: peak I unretained (0.01%); peak I retained (0.03%); BP I (0.01%); BP II (0.07%); BP III (0.07%). Purified BP I, BP II, and BP III also did not react on Western blots to antibodies against C3a des-Arg, α light chain, prealbumin, apolipoprotein A-I, sterol carrier protein-2, or protein 422, a basic 15-kDa fatty acid binding protein from adipose tissue (8, 28).

Fibroblasts—After informed consent was obtained, fibroblasts were grown from skin biopsies taken from the forearm as described (8). Cells were used between passages 9 and 10.

Antibodies—Mouse monoclonal anti-phosphotyrosine antibody, anti-Erk-2 (MAPK R2) monoclonal antibody, and specific antibodies to platelet-derived growth factor receptor, the insulin receptor, and the insulin receptor substrate 1 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Protocol for Cell Experiments—Fibroblasts (10⁵ or 10⁶ cells) were seeded and grown in minimal essential medium containing 10% (v/v) fetal calf serum, 1% amino acids, 100 units/ml penicillin, and 100 mg/ml streptomycin for 6 days. The medium was then changed to a serum-free, lipid-free medium, supplemented with transferrin (5 μg/ml), biotin (8 μg/ml), calcium pantothenate (8 μg/ml), insulin (2.5 μg/ml), triiodothyronine (5 μg/ml), and hydrocortisone (17 ng/ml) (DMEM/F12) (29) and incubated for 24 h (8). The cells were then either harvested for studies of proteins in the basal condition, or some experiments, oleate/albumin (4.6:1, 10 nmol/liter oleate) was added to the medium without BP (control cells) or to medium to which 6 μg/ml BP II was added. The cells were then incubated for 6 h (8–12) and processed for either assessment of immunoochemically reactive activity of cell protein or for fluorescence microscopy. Several experiments were performed in the presence or absence of sodium vanadate (20 μM, an inhibitor of tyrosine phosphatases) (30, 31).

Preparation of Cell Extracts—Normal or hyperapoB fibroblasts (10⁵ cells) were seeded into 75 cm² flasks and grown as described above under cell culture conditions. Fibroblasts were then washed twice with PBS (Dulbecco’s phosphate-buffered saline, pH 7.1, without CaCl₂ and without MgCl₂·6H₂O), scraped into PBS, transferred to tubes, and pelleted by centrifugation. The supernatant was removed, and the pelleted cells were then lysed in ice-cold buffer A (14 mM Hepes, pH 7.0, 140 mM NaCl, 0.1 mM MgCl₂, 10 mM EGTA, 0.5% Triton X-100, 1 mM sodium vanadate, 1 mM 4-2-aminoethyl-benzene sulfonofluoride, 50 μg/ml leupeptin, and 20 μg/ml of the following protease inhibitors: chymostatin, antipain, and pepstatin (32). The lysed cells were frozen at −80 °C until used. After thawing, the lysed cells were sonicated for 20 s, and the lysates were centrifuged in a Beckman TL-100 ultracentrifuge, using a Beckman TLA 100.2 rotor at 65,000 × g for 30 min at 4 °C (32). The protein was measured on each lysate and the supernatant. Protein content was normalized to the lowest protein concentration in a total volume of 500 μl. Cell lysates were frozen (soluble protein fraction) and pellets (insoluble protein fraction) were then used for further analysis (see below).

Processing of Pellets for Analysis of Insoluble Proteins by SDS-PAGE and Western Blotting—The precipitated (insoluble) cell protein fraction was suspended in SDS loading buffer, and the protein was normalized to the lowest concentration and dissolved by sonication for 20 s and then boiling for 5 min. Aliquots were used for SDS-PAGE and Western blots (see below).

Processing of Supernatants for Immunoprecipitation and Analysis of Soluble Proteins by SDS-PAGE and Western Blotting—Washed agarose-conjugated anti-phosphotyrosine beads (20 μl) (monoclonal IgG 2 b k from Upstate Biotechnology) were added to 500 μl of the supernatant (soluble) cell protein fraction (see above) (32). The suspension was gently vortexed and rotated overnight at 4 °C. The samples were then centrifuged at 14,000 rpm for 1–2 min. The pelleted agarose beads were washed four times with lysis buffer. The supernatant was removed, SDS loading buffer (25 μl) was added to the agarose beads and boiled for 5 min, the beads were pelleted, and aliquots of the supernatant were used for SDS-PAGE and Western blots (see below).

SDS-PAGE and Immunoblotting—The SDS-PAGE (4–20%) gradient gel was run at 160 V for 1 h. The gel was transferred to nitrocellulose membrane or at 10 V for 1 h. The blot was then placed in a blocking buffer for 1 h (8% nonfat dry milk (w/v) in TBS-T (Tris-buffered saline with 0.05% Tween) (100 ml total)). The blot was then washed thoroughly, and 1:1000 anti-phosphotyrosine (mouse monoclonal) antibody in TBS-T (30–50 ml) was then added, and the mixture was rocked for 1 h at 25 °C. The blot was washed again thoroughly, and 1:1000 horseradish peroxidase-labeled anti-IgG in...
TBS-T (30–50 ml) was added and incubated with shaking for 45 min. The blot was then washed thoroughly, and a 1:1 mixture of enhanced chemiluminescence (ECL) (Amersham Corp.) detection reagents was added for 1 min to the blot (20 ml for one gel) (32). The blot was then exposed to film for 20, 10, 5, and 2 s.

Quantitation of Immunoreactivity of Fibroblast Proteins to Anti-phototyrosine Monoclonal Antibodies on Western Blots—The chemiluminescent image was photographed using the Stratagene Eagle Eye II (La Jolla, CA) video camera system on Real Image Acquire. The image was saved as a TIF file and imported into the Scanalytic RFLPscan program (Billerica, MA). A Bio-Rad prestained low molecular weight SDS-PAGE standard was used to reference the size of the individual bands. Unless otherwise indicated, the major band in the negative control cell lysate (no stimulation of TKP) was assigned an arbitrary reference of 100, to assign relative concentrations to the sample bands. The banding pattern in the specific molecular weight ranges was assigned as follows: Group (Gp) I (Mr 97,000–120,000), Gp II (Mr 36,000–55,000), and Gp III (Mr 14,000–35,000). The relative intensity of the bands in each group (Gp I, Gp II, and Gp III) for each study subject were totaled, the data were entered into Jandel’s Sigma Stat (San Rafael, CA), and a t test was performed to determine whether there were significant differences in protein TKP between the normal and hyperapoB cells.

Mass Measurements of Lipids in Fibroblasts—After the medium was removed, the cells were washed, and the lipids were extracted as described (8, 9). Sodium hydroxide (1 M) was added to the cell residue and dried overnight, and the protein was redissolved in H2O and measured by the method of Lowry et al. (33). The data are expressed as μg of triglyceride/mg of cell protein.

RESULTS

Tyrosine Kinase Phosphorylation of Fibroblast Proteins from Normal and HyperapoB Cells—We examined TKP of insoluble cellular proteins (Fig. 1) to determine if the decreased reactivity of the proteins to a monoclonal anti-phosphotyrosine antibody from hyperapoB cells observed in pilot experiments was a general characteristic of these cells and to examine how much variability there may be within the normal cell lines. Six normal and five unrelated hyperapoB cells were examined in three separate experiments over a 2-year period (Fig. 1). The mean values for each cell line were computed to provide an average value for each cell line. The average patterns of TKP of normal fibroblast proteins in Gp II (Mr 36,000–55,000) ranged between 498 and 656 and were higher than those of the hyperapoB fibroblasts, which ranged between 211 and 371. The mean patterns of TKP of normal fibroblast proteins in Gp III (Mr 14,000–35,000) ranged between 767 and 1288, which was higher than those of hyperapoB fibroblasts, which ranged between 299 and 644. Gp I (Mr 97,000–120,000) were not significantly different between the normal and hyperapoB cell lines (not shown).

Statistical Analyses of TKP in Fibroblast Proteins from Normal and HyperapoB Cells—For Gp II, the means were separated by over four S.D. and highly significantly different (Table I). For Gp III, the means were less separated (by two S.D.) but still significantly different (Table I). This smaller difference was related to the greater variability in Gp III than Gp II.
Abnormal Tyrosine Phosphorylation in HyperapoB Fibroblasts

The immunoreactivity of insoluble fibroblast proteins from six normal and five hyperapoB cell lines to an anti-phosphotyrosine antibody on Western blots was quantitated by densitometry of a photograph of the ECL exposed film using a Scanalytics RFPC Scan program (see "Experimental Procedures"). Bio-Rad prestained low molecular weight SDS-PAGE standard was used to reference the size of the individual bands in the sample lines. Three experiments were performed (see Fig. 1). Banding patterns in three molecular weights were assigned, and the concentrations of the bands in Gp I, Gp II, and Gp III were totaled. To assess the tyrosine phosphorylation of the insoluble proteins, the major band in the negative control cell lysate (no stimulation of TKP) was assigned an arbitrary reference value of 100, in order to assign relative concentrations to the sample bands. Each cell line was studied in more than one experiment, and the values were averaged. A t test (Jandel's Sigma Stat) was used to test for significant differences for Gp I, Gp II, and Gp III. S.D. values are shown in parentheses.

Tyrosine-phosphorylated proteins.

TKP in HyperapoB Family Members and Relation to Plasma LDL ApoB Levels—The relative concentrations of tyrosine phosphorylation in insoluble cellular proteins were also determined from cultured fibroblasts from seven additional family members (one proband, GG, and six first degree relatives of three probands) as described under "Experimental Methods." A significant inverse correlation was found between the degree of phosphorylation of tyrosine in Gp III proteins and the plasma levels of LDL apoB. Inhibition of Phosphotyrosine Phosphatases—The decreased phosphorylation of tyrosine in proteins from the hyperapoB cells may be due to a deficiency in protein tyrosine phosphorylation per se or to enhanced dephosphorylation of tyrosine by phosphotyrosine phosphatases (30), at a concentration (20 μM) twice that previously stated could inhibit phosphotyrosine phosphatases in cell-free systems (31) (see "Experimental Procedures").

Two normal (KC, JO) and two hyperapoB cells (GG, WY) were incubated with sodium vanadate, with and without BP I, BP II, or BP III (Fig. 3). In the normal cells grown in medium without BP, a number of tyrosine-phosphorylated protein bands were visualized on the Western blot; in contrast to the normal cells, there was a notable and generalized decrease in such immunoreactivity in the hyperapoB cells (Fig. 3). In the normal cells, the addition of BP I or BP II did not change this pattern of immunoreactivity; BP III appeared to decrease the pattern to a small degree. In contrast, in the hyperapoB cells, BP II (but not BP I or BP III) notably increased the immunoreactivity of Gp II and Gp III proteins to the anti-phosphotyrosine monoclonal antibody (Fig. 3).

Inhibition of Phosphotyrosine Phosphatases—The decreased phosphorylation of tyrosine in proteins from the hyperapoB cells may be due to a deficiency in protein tyrosine phosphorylation per se or to enhanced dephosphorylation of tyrosine by cellular phosphatases. To address this question, we used sodium vanadate, an inhibitor of phosphotyrosine-specific phosphatases (30), at a concentration (20 μM) twice that previously shown to inhibit phosphotyrosine phosphatases in cell-free systems (31) (see "Experimental Procedures").

Normal cells

| Groups of tyrosine-phosphorylated proteins | Normal cells (n = 6) | HyperapoB cells (n = 5) | p |
|------------------------------------------|----------------------|------------------------|---|
| Gp I (M_r 97,000–120,000)                | 26.1 (13.0)          | 21.2 (22.5)            | 0.66 |
| Gp II (M_r 36,000–55,000)               | 583.7 (80.6)         | 283.8 (65.0)           | 0.0001 |
| Gp III (M_r 18,000–35,000)              | 1,000.8 (206.3)      | 591.4 (175.8)          | 0.007 |

Effect of Human Serum Basic Proteins on Tyrosine Kinase Phosphorylation of Insoluble Fibroblast Proteins—In a separate experiment, TKP of insoluble cellular proteins were compared between a normal and a hyperapoB cell line that had been incubated with DMEM/F12 medium with or without BP I, BP II, or BP III (Fig. 3). In the normal cells grown in medium without BP, a number of tyrosine-phosphorylated protein bands were visualized on the Western blot; in contrast to the normal cells, there was a notable and generalized decrease in such immunoreactivity in the hyperapoB cells (Fig. 3). In the normal cells, the addition of BP I or BP II did not change this pattern of immunoreactivity; BP III appeared to decrease the pattern to a small degree. In contrast, in the hyperapoB cells, BP II (but not BP I or BP III) notably increased the immunoreactivity of Gp II and Gp III proteins to the anti-phosphotyrosine monoclonal antibody (Fig. 3).

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Two normal (KC, JO) and two hyperapoB cells (GG, WY) were incubated with sodium vanadate, with and without BP I, and the immunoochemical reactivity of the insoluble proteins to an anti-phosphotyrosine antibody was determined by Western blots. Vanadate did not reverse the deficiency in TKP in the hyperapoB cells (data not shown).

The stimulatory effect of BP I on triglyceride mass is deficient in hyperapoB fibroblasts (11). We also examined whether inhibition of phosphotyrosine phosphatases may reverse the biochemical defect in acylation stimulation with BP I shown in hyperapoB cells. At base line (control condition), there was no significant difference for the mean mass of cell triglyceride between the normal and hyperapoB cells (Fig. 4). When BP I was added to the control DMEM/F12 medium (no sodium vanadate present), there was a significant stimulation of cell tri-
Abnormal Tyrosine Phosphorylation in HyperapoB Fibroblasts

versus BP I plus vanadate in normal cells. Protein was determined as described under “Experimental Procedures.”

202.1) than in the normal cells (516.4 ± 671.9 S.D.) was 3.7-fold lower in the hyperapoB cells (137.8 ± 19.8). Thus, there was no evidence supporting a difference in the mass of triglyceride by BP I in the normal cells; this effect of BP I was significantly reduced in hyperapoB cells (Fig. 4). The addition of BP I and sodium vanadate together to the normal cells produced a significantly greater mean stimulation of triglyceride formation than that observed with BP I alone (Fig. 4). Also, when sodium vanadate was added to the control DMEM/F12 condition without BP I, the mean mass of triglyceride was significantly higher in the normal than in the hyperapoB cells. However, when sodium vanadate and BP I were added together to the hyperapoB cells, the mean triglyceride level remained significantly less in the hyperapoB than in the normal cells. These data indicate further that vanadate did not reverse the cellular defect in the hyperapoB cells in response to BP I.

Tyrosine Kinase Phosphorylation in Soluble Fibroblast Proteins—The basal levels of tyrosine phosphorylation in the soluble proteins were also examined (see “Experimental Procedures”). As shown in Fig. 5, there was again some variability in the relative concentrations of the TKP proteins. One normal cell (DR) had lower TKP than the other two normals. All but one (CL) of the hyperapoB cell lines manifested lower TKP than the normal cells. The basal levels of tyrosine phosphorylation in the soluble fibroblast proteins (Fig. 5) was then quantitated by densitometry (see “Experimental Procedures”). Data are expressed as mean and one S.D. *, p = 0.04; **, p = 0.02, for normal versus hyperapoB cells. +, p = 0.04 for BP I alone versus BP I plus vanadate in normal cells.

It is possible that Erk-2 in the soluble fraction was associated with another tyrosine-phosphorylated protein that was binding to the agarose-conjugated anti-phosphotyrosine beads without Erk-2 being itself phosphorylated in tyrosine. Alternatively, tyrosine-phosphorylated Erk-2 might have translated from insoluble to soluble protein fractions without changing its phosphorylation status. To test this possibility, the insoluble protein fractions from the same cell lysates were also processed for SDS-PAGE and immunoblotting. Western blots using the monoclonal antibody to Erk-2 were developed and scanned by densitometry (see “Experimental Procedures”). There was no significant difference between the mean ± S.D. relative amounts of the protein component of Erk-2 in the normal 107.3 ± 22.2 and hyperapoB cells 77.7 ± 19.8 (p = 0.16). Thus, there was no evidence supporting a difference in the mass of Erk-2 between normal and hyperapoB cells, nor for a change in subcellular distribution. To determine the specificity of these observations, we also examined other protein-tyrosine kinase molecules. No differences were found between three normal and three hyperapoB cells in the immunoreactivity of insoluble proteins to antibodies to platelet-derived growth factor receptor, the insulin receptor, and the insulin receptor substrate 1 (data not shown).

**DISCUSSION**

This report provides immunohistochemical evidence that links protein TKP to hyperapoB. While the immunological reagents...
employed here are highly specific for phosphorylated tyrosines, the cellular proteins under study are derived from fibroblasts that have undergone a series of experimental manipulations followed by Western blotting and quantitation by densitometry. This complex process leads to intraindividual variability. Despite this variability, we were able to demonstrate a range of tyrosine phosphorylation in normal cells from multiple experiments over 2 years, and show that tyrosine phosphorylation in Gp II and Gp III proteins was deficient in hyperapoB fibroblasts. Given such variability, we are not proposing here that this assay be added as a genetic marker. However, these immunologic data confirm and extend our previous biochemical studies that support the tenet that TKP might be involved in the pathophysiology of hyperapoB.

Cell culture conditions were selected that minimized the effect of growth factors and cytokines on cell TKP. The cells were first grown in fetal calf serum, which contains a full complement of serum proteins and the level of LDL apoB seen in hyperapoB patients (BP II activity). Under the conditions of cell culture used here, this defect may be associated with decreased activity (phosphorylation) of the molecules involved in the MAPK pathway. As well, there may be decreased phosphorylation of phospholipase C-γ, which may lead to decreased production of diacylglycerol and IP₃, and decreased protein kinase C activity. Inhibiting protein kinase C activity prevents the effects of BP I and BP II from being manifested in both normal and hyperapoB cells (10); however, the stimulation of protein kinase C by an analogue of diacylglycerol appears normal in the hyperapoB cells (10).

The experiment with sodium vanadate suggested that the inhibition of phosphotyrosine phosphatases and the low affinity binding of biotinylated BP I to hyperapoB fibroblasts, while biotinylated BP II has a greater affinity for hyperapoB cells than for normal cells (19). It is not known if BP I and BP II are bound to different sites on the same receptor or whether they are bound to distinct receptors. It appears that BP II stimulates tyrosine phosphorylation of Gp II and Gp III proteins, suggesting that it may bind to transmembrane tyrosine kinase receptor.

Our studies also provide evidence for a significant relationship between the degree of tyrosine phosphorylation in Gp III proteins and the level of LDL apoB in plasma. We previously showed that LDL apoB levels in the six unrelated hyperapoB probands and the six normals were significantly related to the acylation-stimulatory activity in cultured fibroblasts for BP I on triglyceride production and for BP II on cholesterol production (9). Taken together, such information suggests, but does not prove, that the cellular deficiency in hyperapoB cells in TKP and in the response to BP I (understimulation of triglyceride production) and to BP II (overstimulation of cholesterol production) are involved in mediating the increased plasma levels of LDL apoB seen in hyperapoB patients (BP II activity would have to be in the liver).

One possibility is that there is a defect in a transmembrane tyrosine kinase receptor for the BP in hyperapoB. Under the conditions of cell culture used here, this defect may be associated with decreased activity (phosphorylation) of the molecules in the MAPK pathway. As well, there may be decreased phosphorylation of phospholipase C-γ, which may lead to decreased production of diacylglycerol and IP₃, and decreased protein kinase C activity. Inhibiting protein kinase C activity prevents the effects of BP I and BP II from being manifested in both normal and hyperapoB cells (10); however, the stimulation of protein kinase C by an analogue of diacylglycerol appears normal in the hyperapoB cells (10).

The experiment with sodium vanadate suggested that the inhibition of phosphotyrosine phosphatases in the normal cells promoted the production of triglyceride, an observation that might be expected if TKP promotes triglyceride formation. For example, inhibition of protein-tyrosine phosphatase by sodium vanadate has been shown to enhance the autophosphorylation of the platelet-derived growth factor receptor following treatment of Ras-transformed NIH 3T3 cells (34). In hyperapoB cells, there was a defect in response to the stimulatory activity of BP I on cell triglyceride mass; however, the addition of sodium vanadate to BP I did not reverse this defect, as one might expect if the defective TKP observed in hyperapoB cells primarily was due to enhanced activity of phosphotyrosine phosphatases.

Taken together, these data indicate that there is cellular abnormality in hyperapoB fibroblasts resulting in deficient protein TKP. This tenet is compatible with our previous data, where inhibition of TKP with genistein decreased the stimulation of triglyceride formation with BP I in normal cells to a level indistinguishable from hyperapoB cells (11). We also found that the normal stimulation of the formation of cholesterol and cholesteryl ester by BP II in hyperapoB cells was inhibited completely by genistein (12). The marked stimulation of tyrosine phosphorylation of Gp II and Gp III by BP II in hyperapoB cells is consistent with the biochemical data; furthermore, genistein prevents the reversal of deficient protein TKP in hyperapoB cells by BP II (data not shown). Inhibition of TKP therefore clearly had divergent effects in normal and hyperapoB fibroblasts, suggesting that a significant component of the biochemical effects of BP I and BP II was mediated through pathways involving TKP.

Preliminary experiments in our laboratory indicate a deficiency of high affinity binding of biotinylated BP I to hyperapoB cells, while biotinylated BP II has a greater affinity for hyperapoB than for normal cells (19). It is not known if BP I and BP II are bound to different sites on the same receptor or whether they are bound to distinct receptors. It appears that BP II stimulates tyrosine phosphorylation of Gp II and Gp III proteins, suggesting that it may bind to transmembrane tyrosine kinase receptor.

Our studies also provide evidence for a significant relationship between the degree of tyrosine phosphorylation in Gp III proteins and the level of LDL apoB in plasma. We previously showed that LDL apoB levels in the six unrelated hyperapoB probands and the six normals were significantly related to the acylation-stimulatory activity in cultured fibroblasts for BP I on triglyceride production and for BP II on cholesterol production (9). Taken together, such information suggests, but does not prove, that the cellular deficiency in hyperapoB cells in TKP and in the response to BP I (understimulation of triglyceride production) and to BP II (overstimulation of cholesterol production) are involved in mediating the increased plasma levels of LDL apoB seen in hyperapoB patients (BP II activity would have to be in the liver).

One possibility is that there is a defect in a transmembrane tyrosine kinase receptor for the BP in hyperapoB. Under the conditions of cell culture used here, this defect may be associated with decreased activity (phosphorylation) of the molecules in the MAPK pathway. As well, there may be decreased phosphorylation of phospholipase C-γ, which may lead to decreased production of diacylglycerol and IP₃, and decreased protein kinase C activity. Inhibiting protein kinase C activity prevents the effects of BP I and BP II from being manifested in both normal and hyperapoB cells (10); however, the stimulation of protein kinase C by an analogue of diacylglycerol appears normal in the hyperapoB cells (10).

The experiment with sodium vanadate suggested that the inhibition of phosphotyrosine phosphatases in the normal cells promoted the production of triglyceride, an observation that might be expected if TKP promotes triglyceride formation. For example, inhibition of protein-tyrosine phosphatase by sodium vanadate has been shown to enhance the autophosphorylation of the platelet-derived growth factor receptor following treatment of Ras-transformed NIH 3T3 cells (34). In hyperapoB cells, there was a defect in response to the stimulatory activity of BP I on cell triglyceride mass; however, the addition of sodium vanadate to BP I did not reverse this defect, as one might expect if the defective TKP observed in hyperapoB cells primarily was due to enhanced activity of phosphotyrosine phosphatases.

Taken together, these data indicate that there is cellular abnormality in hyperapoB fibroblasts resulting in deficient protein TKP. This tenet is compatible with our previous data, where inhibition of TKP with genistein decreased the stimulation of triglyceride formation with BP I in normal cells to a level indistinguishable from hyperapoB cells (11). We also found that the normal stimulation of the formation of cholesterol and cholesteryl ester by BP II in hyperapoB cells was inhibited completely by genistein (12). The marked stimulation of tyrosine phosphorylation of Gp II and Gp III by BP II in hyperapoB cells is consistent with the biochemical data; furthermore, genistein prevents the reversal of deficient protein TKP in hyperapoB cells by BP II (data not shown). Inhibition of TKP therefore clearly had divergent effects in normal and hyperapoB fibroblasts, suggesting that a significant component of the biochemical effects of BP I and BP II was mediated through pathways involving TKP.
The molecular basis of this abnormality in TKP in hyperapoB fibroblasts remains to be determined. The observations here provide a framework within which to examine systematically relationships between cell surface binding of BP I and BP II, intracellular effects on protein phosphorylation and activation of signal transduction molecules, and abnormalities in cell lipid metabolism. It is not known how such an abnormality in protein TKP in hyperapoB cells may be manifested in vivo. Alterations of proliferation or of apoptosis may accelerate the basic atherosclerotic process in hyperapoB, which is already acerbated by increased numbers of small, dense LDL particles.

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