**Annexin VII and Annexin XI Are Tyrosine Phosphorylated in Peroxovanadate-treated Dogs and in Platelet-derived Growth Factor-treated Rat Vascular Smooth Muscle Cells**

Laura Lowe Furget, Katherine Chen, and Stanley Cohen

*From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146*

The intraperitoneal administration of peroxovanadate results in the rapid accumulation of many tyrosine-phosphorylated proteins in the liver and kidney of treated animals. The availability of large pools of tyrosine-phosphorylated proteins derived from normal tissues facilitates the purification and identification of previously unknown targets for cellular tyrosine kinases. Using this procedure, we have thus far identified four proteins in the liver and kidney of peroxovanadate-treated dogs. Two of these, annexin VII and annexin XI, were novel and had not been previously reported to be substrates of tyrosine kinases while the remaining two, ezrin and clathrin, have been reported to be tyrosine phosphorylated in some cell culture systems. In the present study, isolated proteins were identified both by sequence analysis and immunological methods. Annexin VII and annexin XI are present in cultured rat vascular smooth muscle cells and both were tyrosine phosphorylated in response to a physiological ligand, platelet-derived growth factor-BB (PDGF-BB). Furthermore, the extent of tyrosine phosphorylation in response to PDGF-BB was augmented by the co-addition of peroxovanadate to cell cultures. *In vitro* phosphorylation assays showed that PDGF receptor, calcium-dependent tyrosine kinase (CADTK/Pyk-2), Src kinase, and epidermal growth factor receptor all were able to phosphorylate purified annexin VII and XI on tyrosine residues. These findings confirm the usefulness of phosphatase inhibition by peroxovanadate as a tool for identifying previously unknown physiological targets for cellular protein tyrosine kinases.

Although tyrosine-phosphorylated proteins constitute only a very small percentage of the total phosphoprotein content of cells, the phosphorylation and dephosphorylation of specific tyrosine residues plays an important regulatory role in signal transduction, cell cycle control, and differentiation (1). Enhancement of the tyrosine phosphorylation of specific cellular proteins may be induced by treatment with appropriate cell activating ligands (growth factors, hormones, and cytokines). Many of these extracellular ligands directly or indirectly activate specific tyrosine kinases. Alternatively, we have previously reported that the simple intraperitoneal injection of a tyrosine phosphatase inhibitor (peroxovanadate) into mice, in the absence of any added ligand, results within minutes in the appearance of numerous tyrosine-phosphorylated proteins in liver and kidney. These include the EGF-R, insulin receptor, hepatocyte growth factor receptor, SHC, Stat 1a, Stat 1b, Stat 3, Stat 5, phospholipase Cγ, insulin receptor substrate-1, β-catenin, γ-catenin, SHP-1, SHP-2, etc., all of which were identified using antibodies to known tyrosine-phosphorylated proteins (2). These results emphasize the importance of phosphatase activity on the steady-state levels of phosphorysorine in cellular proteins and the extent to which global tyrosine kinase activity is always "on" in the intact animal (2).

In the present study, *in situ* administration of peroxovanadate to dog was used to generate a sufficiently large pool of tyrosine-phosphorylated proteins in liver and kidney to attempt the biochemical isolation and identification of proteins not previously known to be tyrosine phosphorylated. Among the dozens (hundreds?) of phosphorysorine-containing proteins that could be induced in these organs of the intact animal, we now report the identification of four: annexin VII, annexin XI, clathrin heavy chain, and ezrin. Tyrosine phosphorylation of ezrin and clathrin heavy chain has been detected in some cell culture systems (3–5). Since tyrosine phosphorylation of annexin VII and XI has not previously been reported, we present evidence for the physiological relevance of these phosphorylations: PDGF-BB induces the tyrosine phosphorylation of both annexins in rat vascular smooth muscle cells (VSMC) and both annexins are tyrosine phosphorylated *in vitro* by a number of receptor and cytoplasmic tyrosine kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Immobilon-P membranes were from Millipore (Bedford, MA). Pre-stained molecular weight standards were from Life Technologies, Inc. (Gaithersburg, MD). Polyclonal (rabbit) antibody produced against a unique peptide sequence in annexin XI was a generous gift from Dr. C. Towle (Harvard University) and was used for Western blotting (6). We also thank Dr. W. van Vunrooj (University of Nijmegen) for a gift of an antiserum containing annexin XI antibodies from a patient with an autoimmune disease (antibodies to annexin XI can be found in approximately 10% of patients with systemic autoimmune disorders (7)). The patient antibodies were used for annexin XI immunoprecipitation reactions. The following monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY): RC20H (horseradish peroxidase-conjugated anti-phosphotyrosine), annexin VII, ezrin, and clathrin. A second monoclonal clathrin antibody prepared by Dr. J. Ostermann (Vanderbilt) from X22 cell line (ATCC, Manassas, VA) was used for immunoprecipitation. Polyclonal annexin

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‡ Supported in part by United State Public Health Service Training Grant T32 DK07061. Current address: Dept. of Chemistry, Kalamazoo College, Kalamazoo, MI 49006-5295.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, 607 Light Hall, Nashville, TN 37232-0146. Tel.: 615-322-3318; Fax: 615-322-4349.

1 The abbreviations used are: EGF, epidermal growth factor; EGF-R, EGF receptor; Stat, signal transducer and activator of transcription; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RIPA, radiiodimunoprotection assay; VSMC, vascular smooth muscle cells; PDGF-BB, platelet-derived growth factor-BB; PDGF-R, PDGF receptor; SH2, Src homology 2; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Tyrosine Phosphorylation of Annexin VII and Annexin XI

VII antibody was a generous gift from Dr. H. Pollard (Uniformed Service University, Bethesda). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG polyclonal antibodies were obtained from Transduction Laboratories (Lexington, KY) and Cappell (Durham, NC), respectively. Polyclonal antibody to PDGF-R was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody to PKC alpha and beta (Protein A-Sepharose conjugate) were from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody to fak kinase was the gift of Dr. Steven Hanks (Vanderbilt). Pre-swollen DE52 (diethylami- noethyl cellulose) anion exchange resin was obtained from Whatman (Kent, UK). Pre-swollen DE52 was prepared in this laboratory, Recombinant human PDGF-BB and fibroblast growth factor-basic were purchased from Research Diagnostics, Inc. (Flanders, NJ). Angiotensin II, lysophosphatidic acid, and 12-O-tetradecanoylphorbol-13-acetate were gifts from Dr. T. Inagami (Vanderbilt). Insulin was from Squibb-Novio, Inc. (Princeton, NJ). Hepatocyte growth factor was a gift from Dr. R. Harris (Vanderbilt). Liver and kidney tissues from mouse and rat were purchased from Pel-Freez Biologicals (Rogers, AR) or obtained from stock animals in our laboratory. All other reagents were from Sigma.

Treatment of Dogs and Preparation of Tissue—A 10 ml solution of sodium hydroxide was prepared by heating to boiling. Fifteen minutes prior to use, 30% H2O2 was added to the solution to reduce room temperature to a final concentration of 100 mM. The peroxovana- date solution or PBS alone was injected intraperitoneally into anesthe-tized adult dogs at a dose of 5 ml/kg of body weight. Twenty minutes following treatment, dogs were sacrificed and tissues removed, cut into small pieces, and immediately frozen in liquid nitrogen. Dogs (approx-imately 25 kg) used in these studies were obtained from the Surgery Department at Vanderbilt University and were scheduled for euthana-sia following surgical procedures.

Two methods were used for tissue fractionation. In Method A, pro-teins associated with a membrane/particulate fraction in a Ca2+-de-pendent manner were isolated as follows. Ten percent homogenates (wet wt/vol) were prepared in a Ca2+-containing buffer (10 ml Tricine, pH 8.4, 0.1 M NaCl, 2 mM CaCl2) using a Polytron and then centrifuged at 100,000 x g to separate the soluble from the membrane/particulate bound fraction. The pellet was then extracted with one-fourth original volume of an EDTA-containing buffer (5 mM EDTA, 10 mM Tricine, pH 8.4) and again centrifuged to obtain an EDTA-solubilized fraction. In Method B for tissue fractionation, tissues were homogenized in an EDTA-excluding buffer (20 ml Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM EDTA) and centrifuged at 100,000 x g to obtain a supernatant and a pellet. The pellet was extracted with one-half original volume of 0.1 M Na2CO3 to obtain a membrane-associated protein fraction and then with one-half of the original volume of RIPA buffer (20 ml Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 0.25% deoxycholic acid, 1.5 mM EDTA) to obtain the detergent-soluble fraction (intracellular membrane proteins). All buffers contained 1 mM sodium vanadate and 50 mM sodium molybdate to inhibit protein-tyrosine phosphatases during tissue and sample ma-nipulations. Buffers used for tissue homogenization also contained protease inhibitors (CompleteTM Mini, EDTA-free protease inhibitor mixture tablets, Roche Molecular Biochemicals, Indianapolis, IN) and iodoacetic acid (5 mM) to inhibit sulfhydryl-containing proteases.

Ion Exchange Column Chromatography—A 5-ml bed volume (1 x 6.5 cm) of DE52 resin in Tricine buffer (10 mM Tricine, pH 8.4) was loaded with 20 ml of EDTA-extractable proteins obtained by Method A described above. The flow-through was collected. The column was then washed with 6 ml of Tricine buffer. Two linear gradients were used to elute bound proteins. In the first 15-ml gradient, Tricine buffer containing 0 to 0.2 M ammonium acetate was used for elution. The second 15-ml gradient contained 0.2 to 1 M ammonium acetate in Tricine buffer. A total of 36 fractions of ~1 ml each were collected. All buffers contained 1 mM sodium vanadate and 50 mM molybdate.

Anti-phosphotyrosine Affinity Chromatography—An anti-phospho-tyrosine-agarose column was prepared using 50 ml of packed gel. The column was equilibrated with a Hepes buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM EDTA) and then loaded with two triple loads (~3 mg protein per fraction, 6–13, 14–21, 22–30) from the DE52 column (Fig. 2) dialyzed overnight against 20 mM Hepes buffer, pH 7.4. The samples were then concentrated to ~1 ml using Centricon 30 microconcentrators from Amicon (Beverly, MA). The concentrated fractions were then applied to the column and the flow-through collected and reapplied to the column 20 times. After washing the column three times with 1 ml of Hepes buffer, the phosphotyrosine proteins were eluted with 100 μl of 10 mM phosphotyrosine plus 10 mM methyl phosphate in Hepes buffer and analyzed by SDS-PAGE. All buffers contained 100 μM sodium vanadate. Phospholipid Binding—Phospholipid vesicles (7.5 mg/ml) were prepared as described previously using a phosphatidylserine:cholesterol ratio of 2.5:5 mg/ml (8, 9). Five-hundred microliters of sample from each column fraction containing annexin VII and XI were mixed with lipid vesicles and then applied to a phosphatidylserine:cholesterol vesicle preparation were mixed and left to stand at 4°C for 15 min. After ultracentrifugation at 150,000 x g for 15 min at 4°C, the supernatant was removed and mixed with a fresh 15-μl aliquot of phospholipid vesicles and 5 mM Ca2+ (final). The mixture was incubated for 45 min at 4°C followed by ultracentrifugation at 100,000 x g for 15 min at 4°C. The resulting pellet was washed, re-centrifuged, and ad- sorbed proteins were analyzed by SDS-PAGE.

Western Blotting and Immunoprecipitation—For Western blotting, samples were separated by SDS-PAGE, transferred to Immobilon-P membranes, and probed with antibodies as indicated in the figure legends. Antibody binding was detected by incubation of the mem-branes with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody followed by ECL, except in the case of anti-phosphotyrosine blots where the primary antibody (RC20H1) is conjugated directly to horseradish peroxidase.

For immunoprecipitation reactions, aliquots (200–500 μl) of tissue samples from dog kidney were prepared by Method A or Method B. Samples were incubated with 3 μl of monoclonal clathrin or monoclonal annexin VII antibodies for 2 h followed by addition of 3 μg of anti-mouse IgG-agarose for 30 min. For immunoprecipitation of annexin XI, the samples containing annexin XI were incubated with tissue sample for 2 h followed by addition of 50 μl of protein A-agarose for 1 h. The remaining steps of the immunoprecipitation reactions were performed as described previously (2).

Amino Acid Sequence Analysis—Coomassie Blue-stained purified proteins obtained from slices of Immobilon-P membrane were submit- ted to the Vanderbilt Protein Chemistry Core facility for amino acid sequence analysis. Proteins were directly sequenced on a PE Applied Biosystems Procise 492 Protein Sequencer (Foster City, CA). If direct N-terminal sequencing was not successful, protein from slices of Immobilon-P was digested with endoproteinase Lys-C and/or Asp-N. Resulting polypeptide fragments were then fractionated by high performance liquid chromatography and sequenced. Sequences were then checked against peptide data bases to match experimental results with existing proteins.

Cell Culture—Rat VSMC were prepared from the thoracic aorta of 12-week-old Harlan-Sprague Dawley rats (Charles River Breeding Labo-ratories) by the explant method and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum as described previously (10). Cells (passages 3–15) at 80% confluence in 100-mm dishes were made quiescent by incubation with serum-free Dulbecco’s modified Eage-l’s medium for 24 h prior to treatment with indicated ligands or peroxanadate.

After treatment with the indicated stimuli, cells were washed and then lysed by passage through a 21-gauge needle 20 times. The lysis buffer (1 ml/dish) contained 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM EDTA, 1 mM sodium vanadate, 50 μM molybdate, and protease inhibitors as described above. Following ultracentrifugation for 30 min, the supernatant from the lysate was mixed with phospholipid vesicles (30 μl) and 10 mM Ca2+ for 45 min at 4°C. Samples were then centri-fuged again and the pellet analyzed by SDS-PAGE and Western blotting as described above.

In Vitro Phosphorylation Assays—In vitro kinase assays were performed with purified annexin VII and XI and selected kinases. Annexin VII and XI were purified together from control dog kidney by ion exchange chromatography (as described above) followed by a 25% am-mmonium sulfate precipitation and a second 50% ammonium sulfate precipitation containing annexin VII and XI. The purified annexin VII and XI were essentially free of other proteins (as judged by Coomassie Blue staining). Kinase assays were performed with Src kinase, CADTK/Pyk-2 kinase, JAK2 kinase, Fak kinase, PDGF-R kinase, and EGF-R kinase in buffer containing 20 mM Hepes, pH 7.4, 20 mM MgCl2, 1 mM MnCl2, 100 μM VO43-, 1 mM dithiothreitol, and 5 mM ATP. Mixtures of substrate (~50 ng/μl of each phosphoprotein) and ATP were incubated at room temperature for 15 min prior to centrifugation and analysis by SDS-PAGE. For experiments with CADTK/Pyk-2 kinase, PDGF-R, and Fak kinase, the kinases were immunoprecipitated from homogenates of control dog kidney and bound to Protein A-Sepharose. After incubation with substrate and ATP, the Protein A-Sepharose and attached antibodies was removed by centrifugation to eliminate immunoglobulin heavy chains from the reaction mixture prior to SDS-PAGE.
Tyrosine Phosphorylation of Annexin VII and Annexin XI

Intraperitoneal Injection of Peroxovanadate in Dog Results in Tyrosine Phosphorylation of Multiple Proteins in Liver and Kidney—In previous experiments, the mouse system proved quite successful for the identification of tyrosine-phosphorylated proteins in the peroxovanadate-treated intact animal, using antibodies against known tyrosine kinase substrates (2). However, this system has limitations for protein purification and the isolation of novel substrates due to the small size of the animal and organs. For this reason, tyrosine-phosphorylated proteins were isolated from the liver and kidney of peroxovanadate-treated dogs, thus providing hundreds of grams of tissue for bulk isolation of phosphotyrosine-containing proteins by standard biochemical methods and identification by sequence analysis.

Adult dogs were treated by intraperitoneal injection of PBS alone or PBS containing 10 mM sodium vanadate and 100 mM H₂O₂ as described under “Experimental Procedures.” After 20 min, the liver and kidneys were excised and frozen in liquid nitrogen. Extracts were prepared and the proteins separated by SDS-PAGE and analyzed by Western blotting with antiphosphotyrosine antibodies. In both organs the administration of peroxovanadate resulted in tyrosine phosphorylation of many proteins. Similar preparations from control animals (treated with PBS alone) showed only trace levels of tyrosine-phosphorylated proteins (Fig. 1) in previous experiments with mice, treatment with vanadate or H₂O₂ alone induced only minimal tyrosine phosphorylation in liver and kidney (2).

Isolation and Identification of Tyrosine-phosphorylated Proteins from Liver and Kidney of Peroxovanadate-treated Dogs—Two procedures (Method A and B) were used for initial tissue fractionation. The first method (Method A) was used to obtain those proteins that reversibly associate with a membrane/particulate fraction of the tissue in a calcium-dependent manner. This procedure was previously used to identify tyrosine-phosphorylated annexin I in A431 cells (11). Method B yielded three separate fractions: cytosolic, alkali-extractable (membrane-associated proteins), and detergent-extractable (intrinsic membrane proteins).

The EDTA-extractable fraction (obtained by Method A) from both liver and kidney were analyzed by SDS-PAGE and found to contain multiple tyrosine-phosphorylated proteins as detected by Western blotting with anti-phosphotyrosine antibodies. The results with kidney extracts are shown in Fig. 2, lane labeled “orig.” The proteins present in the EDTA-extractable fractions were then separated by DE52 column chromatography. Fractions were analyzed by SDS-PAGE and Western blotting using anti-phosphotyrosine antibodies (Fig. 2). Many tyrosine-phosphorylated proteins were detected throughout the gradient (Fig. 2, column fractions 5–36). Selected fractions containing tyrosine-phosphorylated proteins were further purified by two procedures: 1) adsorption on anti-phosphotyrosine-agarose and elution with phenyl phosphate and phosphotyrosine, or 2) adsorption on phospholipid-containing vesicles in the presence of calcium and elution with EDTA (see “Experimental Procedures”). The latter procedure is a general method for isolating members of the annexin family of homologous proteins that bind phospholipids in the presence of calcium (12). The samples recovered from each procedure were subjected to SDS-PAGE, transferred to Immobilon-P, and stained with Coomassie Blue. Phosphotyrosine-containing protein bands were excised from the membrane and submitted for sequence analysis in the Protein Chemistry core facility at Vanderbilt.

By these procedures, four tyrosine-phosphorylated proteins (~47, ~56, ~81, and ~180 kDa) were identified. The N-terminal sequence of the 81-kDa protein (isolated from pooled fractions 14–21, Fig. 2) was found to be PKPINVRVTXDK. This sequence corresponds to the N terminus of ezrin. Ezrin has been previously shown to be tyrosine phosphorylated on at least two tyrosine residues (3, 13).

The sequences of a peptide derived from a Lys-C digestion of the ~180-kDa protein (isolated from pooled fractions 22–30, Fig. 2) was determined to be (K)ADDPSXXMEV. This peptide occurs in clathrin. Two peptides were sequenced from the 56-kDa protein (isolated from pooled fractions 6–13, Fig. 2): an Asp-N-derived peptide with the sequence DMTLVQR(D) and a Lys-C-derived peptide with sequence (K)SLYHDIXGD. These peptides correspond to those present in annexin XI. Finally, sequences derived after Lys-C digestion of the 47-kDa protein (also isolated from pooled fractions 6–13, Fig. 2) were (K)GFHTDQAIV and (K)LLLQIVGQ, corresponding to sequences present internally and at the C terminus, respectively, of annexin VII.

Immunoprecipitation and Western Blotting to Confirm the Identification of the Tyrosine-phosphorylated Proteins—To confirm the protein identifications derived from sequence analysis, extracts were immunoprecipitated with specific antibodies against clathrin, annexin VII, and annexin XI and their phosphotyrosine content was detected by Western blotting with antibodies to phosphotyrosine. By these procedures we confirmed that all three proteins were tyrosine phosphorylated (Fig. 3). Furthermore, after incubation of the proteins with leukocyte antigen-related protein, protein-tyrosine phosphatase followed by SDS-PAGE and transfer of the proteins to Immobilon-P, all or most of the signal in Western blots with anti-phosphotyrosine was removed, again indicating that the proteins were tyrosine phosphorylated (data not shown).

The four identified proteins were isolated from an EDTA-extractable fraction indicating that at least a portion of all four proteins were in some manner associated with the membrane/particulate fraction in a Ca²⁺-dependent manner (clathrin light chain, which is associated with clathrin heavy chain, is a Ca²⁺-binding protein (14)), these proteins could also be detected by Western blotting in tissue fractions isolated by other procedures (Method B; see above and under “Experimental Procedures”). The soluble, alkali-extractable (Na₂CO₃) and detergent-extractable (RIPA) fractions obtained using Method B each contained many tyrosine-phosphorylated proteins (Fig.
Tyrosine Phosphorylation of Annexin VII and Annexin XI

Fig. 2. Anion exchange column chromatography. EDTA-extractable proteins from kidney of peroxovanadate-treated dog were separated by anion exchange using DE52 resin as described under “Experimental Procedures” and 20 μl of each fraction was analyzed. Phosphotyrosine (PY) proteins were detected by Western blotting with anti-phosphotyrosine antibodies after SDS-PAGE and transfer to Immobilon-P. Lane 1, orig shows the original total EDTA-extractable proteins. Lane 2, FT, shows the flow-through from the column during sample loading. Proteins were eluted in 1-ml fractions using two linear gradients of 0–0.2 M ammonium acetate and 0.2–1 M ammonium acetate in 10 mM Tricine buffer (Fractions 1–36). The distribution of the four identified proteins among the fractions examined was as expected. Both annexin XI and annexin VII were predominantly in the soluble fraction since the homogenization buffer contained EDTA that would chelate Ca2+ and dissociate the annexins from membranes (Fig. 4, B and C). In addition, an annexin XI immunoreactive band (~42-kDa) was detected in the Na2CO3 fraction (Fig. 4B). However, the protein does not appear to be tyrosine phosphorylated and its identity is not known. Clathrin, as shown by Keen (15), was predominantly in the Na2CO3 fraction (Fig. 4D). Although the major portion of the cytoskeletal protein ezrin was detected in the soluble fraction, some could be detected in all fractions examined (Fig. 4E).

Full-length Annexin XI and Annexin VII Are Absent in Rodent Kidney—In a preliminary experiment, prior to a search for possible physiological ligands that might induce the tyrosine phosphorylation of annexin VII and annexin XI, a comparative examination for the presence of these proteins in liver and kidney tissue from rat, mouse, dog, and human was performed. EDTA-extractable proteins (Method A) from each tissue were analyzed by SDS-PAGE and Western blotting with anti-annexin XI cross-reacting protein (Panel A) or anti-annexin VII (Pollard antibody).

Liver samples from all four species showed full-length annexin XI (Fig. 5A). However, the kidney samples from the four species examined showed marked differences in expression of annexin XI proteins (Fig. 5A). In the rat and mouse kidney samples no trace of full-length annexin XI could be detected (Fig. 5A). The Western blot of the human kidney yielded a weak annexin XI signal (Fig. 5A).

In our screen of kidney and liver from rat, mouse, dog, and human, the 47-kDa isoform of annexin VII was present in liver of all species (Fig. 5B). However, although the 47-kDa form was present in human and dog kidney, we could not detect annexin VII in the kidney of rat and mouse (Fig. 5B). Thus, neither the 47-kDa annexin VII nor the 56-kDa annexin XI could be detected in rodent kidney whereas both were expressed in liver of all species examined.
Tyrosine Phosphorylation of Annexin VII and Annexin XI

**Fig. 6.** Annexin VII and annexin XI are tyrosine phosphorylated in rat VSMC in response to treatment with PDGF-BB, peroxovanadate, and PDGF-BB plus peroxovanadate. Rat VSMC were treated for 5, 10, and 20 min with PDGF-BB (50 ng/ml), peroxovanadate (10 μM vanadate, 50 μM H₂O₂), or peroxovanadate plus PDGF-BB. Lysates from control and treated rat VSMC were incubated with phospholipid vesicles and 10 mM Ca²⁺ for 45 min prior to ultracentrifugation to pellet the phospholipid vesicles and associated proteins that include annexin VII and XI. Samples were then analyzed by SDS-PAGE followed by transfer to Immobilon-P and Western blotting with anti-annexin VII and XI antibodies. Results are representative of at least three experiments; results of all experiments were similar.

In response to treatment of dog with peroxovanadate, a cell culture system was sought in which the effect of known mitogenic stimuli on the tyrosine phosphorylation state of these proteins could be studied.

Mizutani et al. (16) previously found that annexin XI was widely distributed in rat tissues except in the kidney. In addition, they noted that annexin XI was particularly rich in homogenates of the rat aorta (16). Furthermore, we observed, in immunostained sections of dog kidney, that annexin XI was highly expressed in arterial smooth muscle cells.² Given the combination of these findings, we examined cultured rat VSMC by Western blotting with anti-annexin VII and XI antibodies and were able to confirm the presence of these proteins (data not shown).

Treatment of confluent rat VSMC with PDGF-BB (a major mitogenic factor for cultured rat VSMC (17, 18)) or peroxovanadate resulted in an increase in tyrosine phosphorylation of annexin VII and XI as compared with control cells (Fig. 6). In addition, co-addition of peroxovanadate and PDGF-BB resulted in a synergistic increase in tyrosine phosphorylation of annexin VII and XI (Fig. 6). Treatment of rat VSMC with EGF, fibroblast growth factor-basic, hepatocyte growth factor, insulin, 12-O-tetradecanoylphorbol-13-acetate, lysophosphatidic acid, or angiotsin II did not result in detectable tyrosine phosphorylation of annexin VII or XI (data not shown).

An additional protein of molecular mass ~32-kDa showed a strong tyrosine phosphorylation signal in response to treatment of cells with PDGF-BB (Fig. 6). The protein was tentatively identified as annexin II by Western blotting and was expressed at levels ~10–20 times greater than annexin VII and XI (as judged by Coomassie Blue staining; data not shown).

**PDGR-R, CADTK/Pyk-2, Src Kinase, and EGF-R Phosphorylation Annexin VII and Annexin XI on Tyrosine Residues—**Since annexin VII and XI were tyrosine phosphorylated in rat VSMC in response to PDGF-BB, it was of interest to determine which individual protein tyrosine kinase might be responsible and to establish that the annexins are indeed substrates for tyrosine kinases. In **vitro** kinase assays were performed with purified annexin VII and XI and selected kinases. Incubation of purified annexin VII and XI with PDGF-R, CADTK/Pyk-2, Src kinase, or EGF-R in the presence of 5 mM ATP resulted in an increase in phosphorylation of annexin VII and XI on tyrosine residues as indicated by Western blotting with anti-tyrosine antibodies (Fig. 7). Purified annexin VII and XI were shown to be free of contaminating kinase activity by incubation with 5 mM ATP followed by SDS-PAGE and Western blotting with anti-tyrosine antibodies.

**Discussion**

In the present study we have identified in the liver and kidney of peroxovanadate-treated dogs four tyrosine-phosphorylated proteins. Two of these proteins, annexin XI and annexin VII, have not previously been shown to be tyrosine phosphorylated in any model system and the other two, clathrin and ezrin, have not been shown to be tyrosine phosphorylated in the intact animal, although they have been detected in specialized cell systems (3–5, 19).

Annexin VII and XI are related members of a family of homologous proteins that bind negatively charged phospholipids in the presence of calcium. The proposed physiological functions of the annexins include phospholipase A₂ inhibition, exocytosis, membrane trafficking and binding, ion channel activity, and signaling. Although the proposed functions are diverse, they are all related to the phospholipid/membrane binding properties of the proteins.

Annexins are ubiquitously expressed in a variety of tissues and cell types of eukaryotes (20, 21). Family members contain conserved C-terminal domains with conserved repeats of 70 amino acids each of which forms the “endonexin-fold” domain responsible for Ca²⁺ and phospholipid binding. The N-terminal domains are considered unique among annexins since they vary in sequence and length and presumably determine functional diversity among members (20–22). Like eps 8 and eps 15, annexin VII and XI are tyrosine phosphorylated, but contain neither SH2 nor phosphotyrosine-binding domains. Annexin VII and XI both have large, hydrophobic, structurally related N-terminal regions very rich in glycine, tyrosine, and proline residues (20, 21).

The only annexins previously shown to be tyrosine phosphorylated are annexin I and II (20, 21). Annexin I was shown to be tyrosine phosphorylated in A431 cells by the EGF-R in this laboratory (11). Tyrosine phosphorylation of annexin II was detected in cells transformed by pp60⁹⁵ (23). In addition, serine phosphorylation has also been reported for a number of annexins. Despite these findings, the physiological role of phosphorylation of annexins (as well as of annexins themselves) is still largely unknown. Tyrosine phosphorylation may modulate one or more of the proposed physiological functions for this class of proteins.

Annexin VII (also known as synexin) has previously been

² L. Furge, J. McKanna, and S. Cohen, unpublished observations.
shown to occur in two isoforms: one full-length with a molecular mass of 51-kDa and one lacking exon 6 with molecular mass of 47-kDa. In our studies, only the 47-kDa isoform was observed. Annexin VII has been reported to be involved in ion channel activity, membrane fusion, aggregation, secretion, and Ca\(^{2+}\)/GTP-regulated exocytosis (20, 21, 24, 25).

Annexin XI was independently first identified in 1992 by Towle and Treadwell (6) from a bovine chondrocyte cDNA library and by Tukumitsu et al. (26) from rabbit lung as a calcyclin-associated protein. The N-terminal domain has been reported to be responsible for nuclear localization of the protein during rat embryonic development (16, 27, 28) and for antigenic stimulation in human autoimmune disease (7). Mizutani et al. (29) detected serine and threonine phosphorylation, but not tyrosine phosphorylation, of annexin XI in pp60\(^{src}\)-transformed cells.

We were unable to detect either annexin VII or XI in kidney of mouse and rat although both were detectable in the liver of formed cells. We are indebted to T. Fitzgerald for preparing and maintaining rat VSMC cultures and Dr. T. Inagami for use of the Immunology facility (supported in part by United States Public Health Service Grant P30 CA68485) for the amino acid sequencing analysis.

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