Lipocortin 2 (Annexin 2) Is a Major Substrate for Constitutive Tyrosine Kinase Activity in Chondrocytes†

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ABSTRACT: Treatment of cultured bovine articular chondrocytes with 100 μM orthovanadate, in the absence of serum, results in the production of a single major tyrosine phosphorylated protein with an apparent molecular mass of 36 kDa (p36). Chondrocytes were found to contain proteins reactive with anti-lipocortin 1, 2, and 5 antibodies. p36 comigrated on SDS–polyacrylamide gels with lipocortin 2, but not with other members of the lipocortin family. The distribution of p36 between the particulate and soluble cell fractions was also similar to that of lipocortin 2. p36 that was purified on an anti-phosphotyrosine immunoaffinity column cross-reacted with anti-lipocortin 2 antibodies. Similarly, lipocortin 2 purified on an anti-lipocortin 2 immunoaffinity column reacted with anti-phosphotyrosine antibodies. Furthermore, cyanogen bromide cleavage fragments of purified lipocortin 2 and p36 were similar. These data demonstrate that the major constitutively tyrosine phosphorylated protein, in chondrocytes, is lipocortin 2. Tyrosine phosphorylated p36 required SDS buffers for extraction due to a loss of the tyrosine phosphate group under other solubilization conditions using Triton X-100 or sodium cholate. This study provides a system for the study of the effects of tyrosine phosphorylation on lipocortin 2 function. What role lipocortin 2 plays in chondrocyte biology remains to be determined.

Osteoarthritis is characterized by a loss of articular cartilage, due at least in part, to increased chondrocyte production of matrix metalloproteases, such as collagenase and stromelysin (Pelletier et al., 1983; Howell, 1986). It is well established that articular chondrocytes produce collagenase constitutively or when stimulated by cytokines such as interleukin 1 and tumor necrosis factor (Arned & Dayer, 1990). Apart from data demonstrating that a primary gene response involving *fos* and *jun* expression is required, the signaling events leading to the stimulation or inhibition of collagenase production are not known (Chiu et al., 1989; Schonthal et al., 1988; Trabandt et al., 1992). Previous data from our laboratory demonstrated that increased levels of tyrosine phosphorylated proteins in chondrocytes, achieved either by increasing tyrosine kinase activity with serum or by reducing tyrosine phosphatase activity with the phosphotyrosine phosphatase inhibitor, orthovanadate (Swarup et al., 1982; Owada et al., 1989), correlated closely with a marked inhibition of collagenase production (Cruz et al., 1990). In the absence of tyrosine kinase stimulation by serum, chondrocytes treated with orthovanadate, at levels necessary to inhibit collagenase production, showed increased tyrosine phosphorylation predominantly of a 36-kDa (p36) protein. However, the identity of this protein and its role, if any, in the regulation of collagenase production remain to be determined.

Tyrosine phosphorylation of proteins is known to be involved in signal transduction mechanisms regulating cellular function, such as the action of growth factors on cell replication and on cell transformation by certain retroviruses (Pazin & Williams, 1992). It is possible that p36 is a substrate for constitutive tyrosine kinase activity and that the phosphorylation state of this protein plays a role in the regulation of collagenase production.

The lipocortin family contains two well characterized proteins, with molecular weights similar to that of p36, which serve as substrates for tyrosine kinases. Lipocortin 1 (calpactin 2, annexin 1), a 39-kDa membrane-associated protein, is tyrosine phosphorylated by the EGF receptor/tyrosine kinase (Fava & Cohen, 1984; Pepinsky & Sinclair, 1986), recombinant pp60 c-*src*, recombinant pp50 v-*abl* (Varticovski et al., 1988). It is also phosphorylated by protein kinase A (Varticovski et al., 1988), insulin receptor kinase (Karasik et al., 1988), and protein kinase C (Barnes et al., 1991; Varticovski et al., 1988). Lipocortin 2 (annexin 2, calpactin 1 heavy chain) was first characterized as a substrate for pp60 v-*src* in Rous sarcoma transformed cells (Erickson & Erickson, 1980; Radke et al., 1980; Cooper & Hunter, 1983) and later as an in vitro substrate for insulin receptor kinase (Karasik et al., 1988) and protein kinase C (Barnes et al., 1991; Gould et al., 1986). However, the family of lipocortin proteins and their ability to serve as substrates for tyrosine kinases has not been studied in chondrocytes.

In this study, we examined chondrocytes for lipocortin proteins, and we demonstrate that the major constitutively tyrosine phosphorylated protein (p36), in chondrocytes, is lipocortin 2.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies to lipocortin 1, 2, 4, and 5 were produced as described by Pepinsky (1991). Anti-phosphotyrosine antibody was produced in rabbits in our

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laboratory by a method previously described by Kamps and Sefton (1988). Human recombinant interleukin 1β (IL-1) was generously supplied by Ciba-Geigy, Basel, Switzerland. The cDNA probe for human collagenase was from Dr. H. Ponta, Kernforschungszentrum Karlsruhe, Institute Fur Genetik, Karksruhe, Germany. Alkaline Phosphatase conjugated goat anti-rabbit antibodies and the chromatography columns were purchased from Bio-Rad. The alkaline phosphatase reaction kit was purchased from Promega. Highly purified BSA was acquired from Boehringer Manheim. Protein A Sepharose CL-4B was purchased from Pharmacia. Tissue culture dishes were obtained from Becton Dickson. Antibiotic-antimycotic was purchased from Gibco-BRL. c-*src* was purchased from Upstate Biotechnology Incorporated. Glass wool was purchased from Fisher. All other reagents were of analytical grade.

Methods

Isolation of Bovine Articular Chondrocytes. Bovine articular chondrocytes were isolated by the procedure previously described by Cruz et al. (1990). The cells were plated in 100-mm plates (2.4×10^7) in 12 mL of Hams F12 media containing 3% antibiotics and 5% FBS. The cells were allowed to recover for 24 h at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

Measurement of Collagenase Activity and Expression. Chondrocytes were preincubated in Ham's F12 medium in the presence or absence of 100 μM orthovanadate for 2 h. Following the preincubation, the medium was removed, and the cells were incubated with fresh media in the presence or absence of 100 μM orthovanadate for 24 h. The conditioned medium and the cells were assayed for collagenase activity using an ELISA method and collagen zymograms as described previously (Cruz et al., 1990, 1991).

Collagenase expression was determined by Northern blotting using human collagenase cDNA probes. Chondrocytes were incubated, for 9 h, in the presence or absence of IL-1 (20 ng/mL), with or without 100 μM orthovanadate. When orthovanadate was used a 2-h preincubation was conducted. Following the incubation, chondrocytes were washed with PBS and resuspended in solution D (5.29 M guanidinium isothiocyanate, 30 mM sodium citrate, 0.72% *N*-laurylsarcosine, 0.72% 2-mercaptoethanol). To this solution was added the following: one-tenth volume 2 M sodium acetate, one-tenth volume water saturated phenol, and one-fifth volume chloroform. The solution was mixed well and left on ice for 15 min. After centrifugation at 3780*g*, the aqueous phase was removed and added to an equal volume of isopropanol and placed at −70 °C for at least 1 h. The RNA was then pelleted at 15325*g* and resuspended in cold 80% ethanol. The final pellet was dried and resuspended in diethyl pyrocarbonate treated water and stored at −70 °C. Samples were run on 1% formaldehyde–agarose gels and visualized by ethidium bromide staining to ensure equal loading. The samples were transferred to nylon membranes and examined by Northern blotting with collagenase cDNA probes.

Immunoblots. Western blotting was conducted as described previously in Cruz et al. (1990) using antibodies to ptyr (1:750) or lipocortin 1, 2, 4, or 5 (1:1000).

Separation of Soluble Cytoplasmic Proteins. Chondrocytes (2.4×10^7) were pretreated for 2 h followed by incubation with 100 μM orthovanadate and 5% FBS for 24 h. The conditioned medium was removed, and the cells were washed two times with cold PBS containing 100 μM orthovanadate and then scraped in 1 mL of the same buffer and centrifuged at 4 °C for 10 min at 15320*g*. The pellet was resuspended in 200 μL of PBS containing 100 μM orthovanadate and lysed by one cycle of freeze-thawing. The soluble fraction containing the cytosolic proteins was separated by centrifugation at 15320*g* for 10 min, at 4 °C. An equal volume of Laemmli buffer containing 100 μM orthovanadate was added to the soluble fraction. The pellet was solubilized in a volume of Laemmli buffer equal to the total volume of the cytoplasmic sample. The proteins from both samples were analyzed by SDS–PAGE and Western blotting with antibodies to ptyr or lipocortin 1, 2, 4, or 5.

Detergent Solubilization of Chondrocyte p36 and Lipocortin 2. Chondrocytes (2.4×10^7) were preincubated for 2 h followed by incubation for 24 h, in Ham's F12 media supplemented with 5% FBS and 100 μM orthovanadate. The cells were washed twice with cold PBS containing 100 μM orthovanadate and harvested in 1.0 mL of (a) 1.0% or 1.5% Triton X-100 in PBS, (b) 0.5% or 1.0% sodium cholate in PBS, or (c) 0.5% or 1.0% SDS in PBS. The samples were mixed on a rocker for 10 min at 4 °C, followed by centrifugation at 15320*g* for 10 min, at 4 °C. An equal volume of Laemmli buffer was added to the supernatants, and the samples were boiled for 5 min. The pellets were solubilized in a volume of Laemmli buffer equal to the final volume of the supernatant samples. The proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose blots. Lipocortin 2 and p36 were visualized by Western analysis with antibodies to lipocortin 2 or ptyr.

Preparation of Immunoaffinity Chromatography Columns. One gram of Protein A Sepharose was soaked in 0.1 M HCl and washed two times with 0.05 M diethylamine, pH 11.5, and three times with PBS, pH 8.0. An equal volume of antiserum to phosphotyrosine or lipocortin 2 was mixed with the Sepharose beads on a rocker for 30 min at 4 °C. The beads were washed three times with PBS and two times with 0.02 M triethanolamine, pH 8.6, and resuspended in 30 mL of triethanolamine. To the solution was added 194 mg of dimethyl pimelimidate dihydrochloride. The solution mixed for 30 min on a rocker at 4 °C, following which 30 mL of 1 M ethanolamine, pH 8, was added. Following 10 min of mixing at 4 °C, the beads were washed three times with 50 mM sodium borate, pH 8.2. Immunoaffinity chromatography columns with bed volumes of 0.5 mL were assembled for the purification of tyrosine phosphorylated protein or lipocortin 2.

Isolation of Tyrosine Phosphorylated Proteins. Orthovanadate treated cells were washed two times with cold PBS containing 100 μM orthovanadate. The cells were harvested in 1 mL of cold PBS containing 100 μM orthovanadate and centrifuged for 10 min at 15320*g* at 4 °C. The pellet was solubilized with 1.0 mL of 0.125% SDS containing 100 μM orthovanadate per 2.4×10^7 cells collected, and the sample was mixed for 10 min at 4 °C and then boiled for 5 min. Insoluble cellular material was pelleted by centrifugation at 15320*g* for 20 min at 4 °C. The supernatant was passed through a chromatography column tightly packed with glass wool, to remove any remaining insoluble particles. The soluble cell extract was diluted 1:1 with PBS containing 100 μM orthovanadate and loaded onto an anti-phosphotyrosine (anti-ptyr) immunoaffinity chromatography column. The unbound proteins were removed by washing the column with 20 column volumes of cold PBS containing 100 μM orthovanadate. Proteins bound to the column were eluted with 5 mL of 40 mM phenyl phosphate containing 200 μM sodium orthovanadate, 90 mM NaCl, 0.05% SDS, and 1 mM NaF, pH 8.0, and collected in 0.5-mL aliquots.

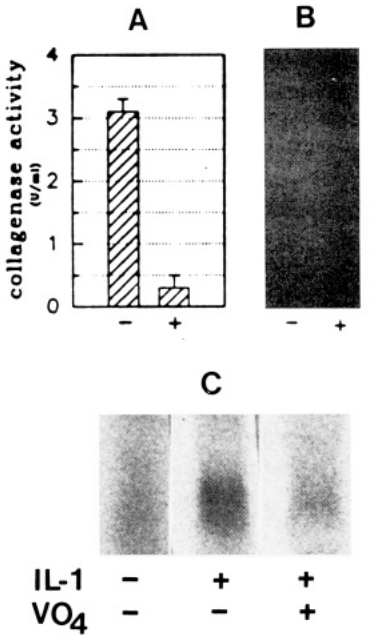

FIGURE 1: Effect of orthovanadate on collagenase activity and mRNA levels. Chondrocytes (2.4×10^7) were incubated in Ham's F12 media in the presence (+) or absence (–) of 100 μM orthovanadate. Following a 24-h incubation, the conditioned medium was removed and analyzed for collagenase activity using (a) an ELISA method and (b) collagen zymograms. (c) Chondrocytes (2.4×10^7) were incubated in Ham's F12 media or media containing IL-1 in the presence or absence of 100 μM orthovanadate, for 9 h. Cellular RNA was isolated and the levels of collagenase mRNA determined by Northern blotting as described under Materials and Methods.

An equal volume of Laemmli buffer containing 100 μM orthovanadate was added to the eluted fraction, and the samples were boiled for 5 min. The proteins were separated on a 12% SDS–polyacrylamide gel and analyzed for lipocortin 1, lipocortin 2, and phosphotyrosine by Western analysis as described above.

Isolation of Lipocortin 2. Soluble cell extract, prepared as described above, was loaded onto an anti-lipocortin 2 immunoaffinity column. The column was washed with 20 column volumes of cold PBS containing 100 μM orthovanadate to remove unbound proteins. Bound proteins were eluted with Laemmli buffer containing 100 μM orthovanadate, and the sample was boiled for 5 min. The proteins were separated on a 12% SDS–polyacrylamide gel and examined by Western analysis.

Cyanogen Bromide Mapping. p36 purified from bovine chondrocytes using immunoaffinity chromatography and lipocortin 2 purified from bovine lung by conventional methods were subjected to cyanogen bromide mapping on 10–20% SDS–polyacrylamide gradient gels as described by Pepinsky (1991). Lipocortin 2 from bovine lung was phosphorylated by c-*src* prior to analysis. Cleavage products were transferred to PVDF and visualised by Western blotting with anti-lipocortin 2 or anti-phosphotyrosine antibodies or by autoradiography.

RESULTS

Effect of Orthovanadate on Collagenase Production and Tyrosine Phosphorylation in Chondrocytes. As demonstrated in Figure 1 panels a and b, bovine chondrocytes in monolayer culture produce collagenase constitutively. Media from chondrocytes treated with 100 μM orthovanadate had much lower collagenase activity. Similar data were obtained by measuring collagenase activity using collagen zymograms.

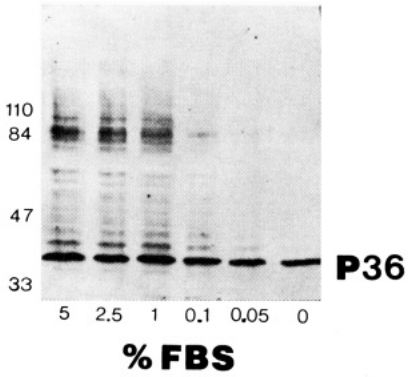

FIGURE 2: Effect of orthovanadate on phosphotyrosine levels. Chondrocytes (2.4×10^7) were incubated with 100 μM orthovanadate, in the presence of 0, 0.05, 0.1, 1.0, 2.5, and 5.0% FBS. Following a 24-h incubation, the cells were solubilized by the addition of 1.0 mL of Laemmli buffer supplemented with 100 μM orthovanadate. The proteins were separated on a 12% SDS–polyacrylamide gel and analyzed by Western blotting using anti-ptyr antibodies.

These experiments were carried out in the absence of serum, since FBS is known to inhibit constitutive collagenase production.

Decreases in collagenase activity in the media could be due to several causes including decreased gene expression, decreased protein synthesis, or inhibition of secretion. An inhibition of procollagenase secretion by orthovanadate is unlikely since no increase in collagenase activity, within treated chondrocytes, was detected (data not shown). To investigate orthovanadate's effect on collagenase gene expression, we stimulated collagenase expression with interleukin-1 (Figure 1c), since constitutive collagenase mRNA levels are low. Under these conditions we found that orthovanadate, at concentrations necessary to elevate cellular ptyr levels, was able to reduce IL-1 induced collagenase mRNA levels. We can eliminate a general decrease in protein synthesis as the cause of orthovanadate's effects on collagenase production, since 100 μM orthovanadate does not significantly affect the amount of proteoglycans produced by cultured bovine chondrocytes (Cruz et al., 1990). Overall, it can be concluded that orthovanadate inhibits constitutive and IL-1 induced chondrocyte production of collagenase at the level of collagenase gene expression.

Since orthovanadate is a potent inhibitor of tyrosine phosphatases, we examined the effect of orthovanadate on cellular ptyr levels (Figure 2). Several concentrations of FBS were used (0, 0.05, 0.1, 1, 2.5, and 5%) to determine the optimum conditions for p36 tyrosine phosphorylation. Although p36 was the major tyrosine phosphorylated protein in the absence of FBS, addition of FBS resulted in increased tyrosine phosphorylation of cellular proteins, including p36. This is due to growth factors present in FBS that stimulate tyrosine kinase activity.

Distribution of p36 and Lipocortins in Chondrocytes. Two members of the lipocortin/annexin family with molecular weights similar to that of p36 have been shown to serve as substrates for various tyrosine kinases. Chondrocytes were examined for the presence of lipocortin 1, 2, 4, and 5. In addition, the distribution, between the soluble and particulate cell fractions, of the lipocortins present was determined as described under Materials and Methods. Chondrocytes were found to contain proteins reactive with lipocortin 1, 2, and 5 antibodies, but not with lipocortin 4 (Figure 3). Lipocortin 5, which appeared as a doublet, reflecting the products from two different bovine lipocortin 5 genes, and lipocortin 1 were present in both the cytosolic and particulate fractions.

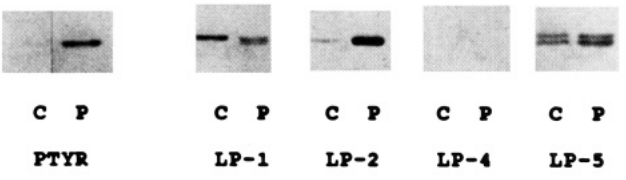

FIGURE 3: Cellular distribution of p36 and lipocortins 1, 2, 4, and 5. Chondrocytes (2.4×10^7) were incubated for 24 h, in the presence of 100 μM orthovanadate, in Ham's F12 media supplemented with 5% FBS. The cells were harvested, and the cytosolic (C) and particulate (P) fractions were separated, as described under Materials and Methods. The proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose blots. Proteins reactive with antibodies to lipocortin 1 (LP-1), lipocortin 2 (LP-2), lipocortin 4 (LP-4), or lipocortin 5 (LP-5), as well as to antibodies to ptyr, were identified by Western analysis, as described under Materials and Methods.

FIGURE 4: Migration of lipocortin 1, lipocortin 2, and p36. Chondrocytes (2.4×10^7) were incubated for 24 h in Ham's F12 media supplemented with 5% FBS and 100 μM orthovanadate. The cells were harvested and solubilized in 0.125% SDS as described under Materials and Methods. The proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose blots. Each transferred sample was divided in two lengthwise, with one half being reacted with anti-phosphotyrosine antibody and the other with antibody to either lipocortin 1 (LP-1) or lipocortin 2 (LP-2), as described under Materials and Methods.

Lipocortin 2 and p36 had a similar distribution, being found predominantly in the particulate fraction.

Migration of Lipocortin 1, Lipocortin 2, and p36 on SDS–Polyacrylamide Gels. Lipocortin 1, lipocortin 2, and p36 migrate similarly on SDS–polyacrylamide gels. To further compare their migration, cell extracts from orthovanadate treated chondrocytes were run in wide gel lanes and the proteins transferred to nitrocellulose blots. Each transferred sample lane was cut in half lengthwise. Each half was analyzed by Western blotting using anti-ptyr antibodies or either anti-lipocortin 1 or anti-lipocortin 2 antibodies. Figure 4 shows that lipocortin 2 migrates identically to p36, while lipocortin 1, due to its higher molecular weight, migrates slower than p36.

Detergent Solubilization of p36 and Lipocortin 2. Since both p36 and lipocortin 2 were enriched in the particulate fraction, several common detergent solutions were employed to solubilize p36 and lipocortin 2 (Figure 5). All the buffers tested were able to solubilize lipocortin 2 from the samples. The SDS buffers were also found to solubilize tyrosine phosphorylated p36.

The Triton X-100 (1.0%, 1.5%) and sodium cholate (0.5%, 1.0%) buffers were ineffective in solubilizing p36 in its tyrosine phosphorylated form. This is probably not due to inefficient sample solubilization, since a strong lipocortin 2 signal was observed in the soluble fractions. To clarify whether failure to isolate tyrosine phosphorylated p36 was due to incomplete sample solubilization, the Triton X-100 and sodium cholate pellets were further solubilized in 1% SDS buffer and examined for tyrosine phosphorylated p36. We found little or no p36 signal (data not shown), indicating a loss of the p36 tyrosine phosphate group. It can therefore be concluded that the

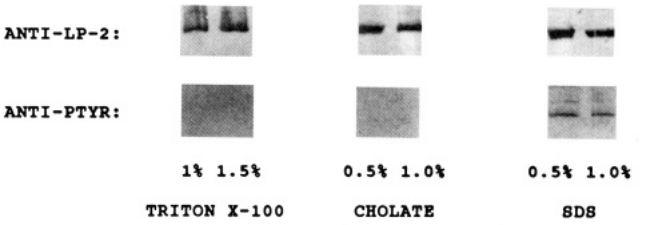

FIGURE 5: Solubilization of chondrocyte p36 and lipocortin 2. Chondrocytes (2.4×10^7) were incubated for 24 h in Ham's F12 media supplemented with 5% FBS and 100 μM orthovanadate. The cells were washed twice with cold PBS containing 100 μM orthovanadate and were harvested in 1.0 mL of detergent/PBS buffer with (a) Triton X-100, (b) sodium cholate, or (c) SDS. The sample was mixed for 10 min at 4 °C. The soluble proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose blots for Western analysis with antibodies to lipocortin 2 and ptyr.

FIGURE 6: Immunoaffinity purification of p36 and lipocortin 2. Chondrocytes (2.4×10^7) were incubated for 24 h in Ham's F12 media supplemented with 5% FBS and 100 μM orthovanadate. The cells were harvested and solubilized in 0.125% SDS as described under Materials and Methods. (a) Tyrosine phosphorylated proteins were isolated from chondrocyte extracts using an anti-ptyr immunoaffinity column. The proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose blots. Each transferred sample was divided in two lengthwise, with one half being reacted with anti-phosphotyrosine antibody and the other with anti-lipocortin 2 antibody, as described under Materials and Methods. (b) Lipocortin 2 proteins from chondrocyte extracts were isolated using an anti-lipocortin 2 immunoaffinity column. The proteins were run on a 12% polyacrylamide gel and transferred to nitrocellulose blots. The proteins were analyzed for ptyr content by Western analysis as described under Materials and Methods.

inability of the Triton X-100 or sodium cholate buffers to solubilize tyrosine phosphorylated p36 was not due to inefficient sample solubilization but was instead due to a loss of the tyrosine phosphate group from p36.

Antibody Reactivity of p36 and Cyanogen Bromide Cleavage Mapping. Tyrosine phosphorylated proteins from orthovanadate treated chondrocytes were isolated by anti-ptyr immunoaffinity chromatography. The samples were run on an SDS–polyacrylamide gel, and found to consist primarily of p36. p36 reacted with anti-lipocortin 2 antibody (Figure 6a), indicating that lipocortin 2 is constitutively tyrosine phosphorylated in chondrocytes and that either it is p36 or it copurifies with p36.

To confirm that lipocortin 2 is constitutively tyrosine phosphorylated, we used anti-lipocortin 2 immunoaffinity chromatography to isolate lipocortin 2. The purified lipocortin 2 reacted strongly with anti-ptyr antibody, confirming that lipocortin 2 is highly tyrosine phosphorylated (Figure 6b).

The identity of the 36 kDa phosphoprotein as lipocortin 2 was verified by CNBr mapping. Under the conditions used, treatment of p36 with CNBr yielded 15 cleavage products that could be detected by Western blotting with the lipocortin 2 antibody (Figure 7, lane A). The same series of products were detected when bovine lipocortin 2 was submitted to the mapping analysis (lane C), providing support that p36 is lipocortin 2. Since it was possible that the 36-kDa band was

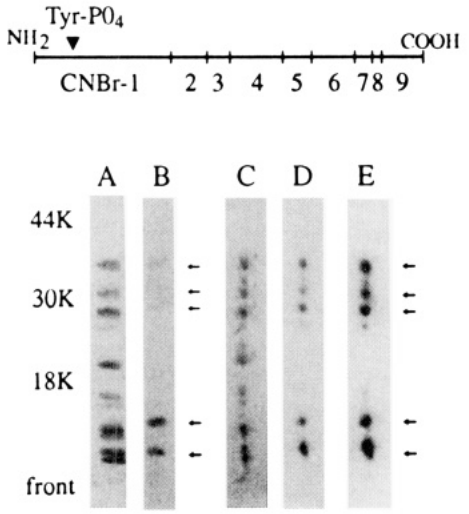

FIGURE 7: Cyanogen bromide cleavage of p36 and lipocortin 2. p36 purified from bovine chondrocytes using immunoaffinity chromatography (lanes A and B) and lipocortin 2 purified from bovine lung by conventional methods (lanes C–E) were subjected to CNBr mapping on 10–20% SDS–polyacrylamide gradient gels as described by Pepinsky (1991). Lipocortin 2 from lung was phosphorylated by c-*src* prior to analysis. Cleavage products were electrophoretically transferred to PVDF and visualized using anti-lipocortin 2 (lanes A and C) or anti-phosphotyrosine antibodies (lane B) or by autoradiography (lanes D and E). The autoradiograph was exposed for 3 (D) or 12 h (E). The positions of the molecular weight standards are indicated at the left of the panel. The schematic at the top of the figure summarizes important structural features of lipocortin 2. Eight internal methionines at positions 117, 149, 170, 216, 239, 277, 291, and 299 dissect the protein at the positions indicated (+). The resulting CNBr fragments are denoted where 1 is the N-terminal fragment and 9 is the C-terminal fragment. Tyrosine-23, the known target of tyrosine protein kinases, is also indicated.

a mixture of products but only lipocortin 2 had been detected with the antisera, we also analyzed the cleavage products for species that were recognized by the anti-phosphotyrosine antibody (lane B). Four cleavage products with apparent masses of 32, 29, 15, and 12 kDa were detected. The same pattern of products was observed when lipocortin 2 that had been phosphorylated by c-*src* was analyzed (lane D). Four phosphorylated fragments with masses of 32, 29, 15, and 12 kDa were also observed. Together these results clearly indicate that the major phosphorylated product in vanadate treated chondrocytes is lipocortin 2. Since lipocortin 2 has eight internal methionines, one would expect to see nine phosphorylated products under limiting digestion conditions with CNBr. CNBr fragment 1 and partial digestion products corresponding to fragments 1+2, 1–3, 1–4, 1–5, 1–6, 1–7, and 1–8 are represented in the digest. When that same blot that is shown in lane D was exposed to X-ray film for four times as long (lane E), we observed eight products. While we do not know the identity of the missing fragment, it is likely that fragment 1–7 and 1–8 would not have been resolved by SDS–PAGE since CNBr fragment 8 is only seven amino acids long.

DISCUSSION

In this study, we have determined that lipocortin 2 is the major substrate for constitutive tyrosine kinase activity in bovine articular chondrocytes maintained in monolayer culture. Both the tyrosine phosphorylated and unphosphorylated forms of lipocortin 2 were enriched in the particulate fractions obtained from the chondrocytes. This is consistent with other studies demonstrating that the free form of lipocortin 2 is localized in the plasma membrane and that the tetramer form, calpactin 1, consisting of two lipocortin 2 proteins bound to a p11 protein dimer, is associated with the membrane cytoskeleton (Saris et al., 1986; Gerke & Weber, 1984; Amiguet et al., 1990; Regnouf et al., 1991; Thiel et al., 1992). Since the particulate fraction contains both the plasma membrane and the membrane cytoskeleton, we were unable to determine the distribution of the tyrosine phosphorylated form between the two structures.

We found that lipocortin 2 could be solubilized from cell extracts with SDS, sodium cholate, and Triton X-100 buffers. Tyrosine phosphorylated lipocortin 2 could be isolated only through rigid adherence to a protocol requiring SDS in the solubilization buffer. The difficulty in isolation of tyrosine phosphorylated lipocortin 2 may not be due to dephosphorylation as would normally be assumed. Tyrosine phosphorylation increases the likelihood of proteolytic cleavage of lipocortin 2's tail region, which contains the tyrosine phosphorylation site and a site susceptible to protease digestion, upstream from the phosphorylation site (Glenney & Tack, 1985; Johnsson et al., 1988). Solutions of Triton X-100 or sodium cholate, though able to solubilize lipocortin 2 from the cells, may have been unable to prevent proteolytic cleavage of the tyrosine phosphorylated tail region, resulting in low yields of tyrosine phosphorylated lipocortin 2 (p36).

The calcium-dependent phospholipid binding activity of lipocortin 2 and the additional binding affinity of the calpactin 1 complex for the cytoskeletal components actin and spectrin (Gerke & Weber, 1984; Regnouf et al., 1991; Ikebuchi & Waisman, 1990; Saris et al., 1986; Pepinsky et al., 1988) has led to speculation that lipocortin 2 may regulate membrane–membrane or membrane–cytoskeleton interactions (Pepinsky et al., 1988; Glenney & Tack, 1985). Studies using permeabilized adrenal chromaffin cells suggest that calpactin 1 may be involved in exocytosis (Ali et al., 1989; Sarafian et al., 1991; Creutz, 1992). This raises the possibility that the decrease in collagenase activity in the media of orthovanadate treated chondrocytes is due to the inhibition of calpactin 1 mediated procollagenase exocytosis. Examination of intracellular collagenase activity and proteoglycan production in the conditioned media indicated that orthovanadate does not decrease protein synthesis or secretion. In addition, orthovanadate is able to inhibit IL-1 induced increases in collagenase mRNA. It is likely that orthovanadate inhibits constitutive collagenase production in chondrocytes in a similar manner, that is, through a decrease in collagenase gene expression.

Keutzer and Hirschhorn (1990) have suggested that calpactin 1 may participate in the transmission of external mitogenic signals through the cytoskeleton to the nucleus. In addition, there exists some evidence that TNF-induced collagenase production, in fibroblasts, may be controlled by signals transmitted to the nucleus via the cytoskeleton (Gronowicz et al., 1992). It is possible that collagenase production in chondrocytes could be regulated through a similar pathway and that calpactin 1 could act as a bridging element in this signal transmission. Regulation could be accomplished through specific phosphorylation and dephosphorylation events (Keutzer & Hirschhorn, 1990), which could alter the ratio of free lipocortin 2 to calpactin 1 or lipocortin 2's distribution within the cell. Phosphorylation of tyrosine residues in the tail region (Tyr-23) alters the calcium/phospholipid binding properties of lipocortin 2 (Schlaepfer & Haigler, 1987; Powell & Glenney, 1987; Glenney & Zokas, 1988) and therefore could affect lipocortin 2's association with membrane phospholipids.

Alternately, increased tyrosine phosphorylation, which increases proteolytic cleavage of the tail region, could decrease

calpactin 1 levels, since the tail region contains the p11 binding site (Amiguet et al., 1990; Johnsson et al., 1988). Tetramer formation is necessary for the association of lipocortin 2 with the membranous cytoskeleton (Thiel et al., 1992); therefore, tyrosine phosphorylation could cause a release of lipocortin 2 from the cytoskeleton. Although our data suggest that tyrosine phosphorylation of lipocortin 2 does not alter its distribution between the cytosolic and particulate fraction, further study is required to determine the fate of tyrosine phosphorylated lipocortin 2.

Shisheva and Shechter (1993) have found evidence of a staurosporine-inhibitable, vanadate-stimulated, cytosolic tyrosine kinase in adipocytes. This raises the possibility that vanadate may stimulate a kinase in chondrocytes that selectively tyrosine phosphorylates lipocortin 2. As of yet, no evidence for the presence of a similar tyrosine kinase in chondrocytes has been produced (data not shown). It is most likely that vanadate's inhibition of tyrosine phosphatases is allowing the visualization of constitutively tyrosine phosphorylated lipocortin 2.

In summary, we have demonstrated that lipocortin 2 is a major substrate for constitutive tyrosine kinase activity in chondrocytes. The role of lipocortin 2, if any, in the regulation of collagenase production awaits further understanding of the signaling events regulating collagenase production and the effects of orthovanadate upon those events.

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