Phosphoprotein Phosphatase Inhibitor-2

IDENTIFICATION AS A SPECIES OF MOLECULAR WEIGHT 31,000 IN RABBIT MUSCLE, LIVER, AND OTHER TISSUES*

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Specific antibodies, raised to purified rabbit skeletal muscle inhibitor-2, were used to analyze for the presence of inhibitor-2 in extracts of rabbit skeletal, cardiac, and diaphragm muscles, liver, kidney, brain, and lung. Western analyses of the extracts by "Western blotting" revealed several immunoreactive species, apparent molecular weights in the range 26,000–136,000, as well as species with the electrophoretic mobility of inhibitor-2, apparent molecular weight 31,000. When supernatants from boiled extracts were similarly analyzed, most of the immunoreactive material was lost and the species corresponding to inhibitor-2 became prominent. Liver and muscle were studied in more detail; immunoprecipitates from either boiled or unboiled extracts were analyzed by Western blotting. The dominant polypeptide now was the species of apparent molecular weight 31,000, corresponding to inhibitor-2. Higher molecular weight species (115,000 in muscle and 136,000 in liver) were also detectable. The amount of inhibitor-2 detected in immunoprecipitates was not greatly different whether unboiled or boiled tissue extracts were used. In addition, extraction of the precipitates by boiling released material that inhibited purified type 1 protein phosphatase. The results suggest that inhibitor-2 is widely distributed in rabbit tissues and is found predominantly as a form of apparent molecular weight 31,000. In particular, the study provides direct demonstration of a species in rabbit liver with similar properties to rabbit muscle inhibitor-2.

Phosphoprotein phosphatase inhibitor-2 (1, 2) is a heat-stable protein, molecular weight approximately 31,000, that inhibits the catalytic subunit of phosphatases of type 1 (3, 4). Almost all chemical information about inhibitor-2 derives from studies of the rabbit skeletal muscle protein, that has been purified to homogeneity in several laboratories (5, 6). Purification has taken advantage of a heat treatment of muscle extracts since the inhibitor-2 resists this step and inhibitors in other tissues is less certain. Studies of liver have indicated the presence of low molecular weight protein inhibitors of phosphatase activity (14–17). The relationship of these species to the better studied muscle inhibitors is not completely clear and in the only case where purification to homogeneity was described, a species of apparent Mr, approximately 15,000 was obtained (15). The conclusion of the present investigation is that inhibitor-2 can be detected mainly as a species of apparent molecular weight 31,000 in rabbit skeletal, diaphragm, and cardiac muscle, liver, brain, lung, and kidney.

EXPERIMENTAL PROCEDURES

Antibodies to Inhibitor-2—Female guinea pigs (460–500 g) were injected subcutaneously with an emulsion of Freund's complete adjuvant (1 ml) containing 80 μg of purified rabbit skeletal muscle inhibitor-2. After 4 weeks, a booster injection was administered and 2 weeks later the animals were bled. Preimmune serum was obtained from heart puncture before inoculation. For some experiments, antibodies were purified from serum by affinity chromatography. Purified inhibitor-2 (0.8 mg) was coupled to 1 g of CNBr-activated Sepharose 4B (see Ref. 18). Immune serum (6 ml) was applied to a column (2.5 ml) of inhibitor-2-Sepharose. After washing with 20 mM Tris-HCl, pH 7.0, plus 0.1 mM NaCl and then 20 mM Tris-HCl, pH 7.0, plus 0.45 M NaSCN, specific antibodies were eluted with 3 M NaSCN and then dialyzed against 10 mM sodium phosphate, pH 7.4, plus 0.13 M NaCl.

Preparation of Tissue Extracts—Rabbits (male New Zealand White, 2.5–3.5 kg) were sacrificed by injection of sodium pentobarbital in the marginal vein of the ear. Portions of the following tissues were excised: skeletal, diaphragm and cardiac muscle, liver, kidney, brain, and lung. These were frozen in liquid nitrogen and stored at −70 °C until processing. Tissue was homogenized in a ratio of 1 g/ml of buffer, pH 7.2, composed of 50 mM Tris-HCl, 2 mM EGTA, 5 mM EDTA, 100 mM NaF, 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM N'-tosyl-L-lysine chloromethyl ketone HCl, 0.2 mM 1-1-tosylamide-2-phenylethyl chloromethyl ketone, and 2 μg/ml of leupeptin. After disruption using a Polytron, setting 9 for 30 s, the homogenate was centrifuged for 15 min at 13,000 x g and the supernatant was removed. A portion was retained (unboiled extract) while another was heated to 100 °C for 10 min before centrifugation as above to yield a supernatant (boiled extract).

Immunoprecipitation of Inhibitor-2 from Muscle and Liver Extracts—Boiled or unboiled extracts (250 μl) of liver or skeletal muscle,

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1 g of tissue/5 ml of buffer for these experiments, were incubated with 19 \( \mu \)g of affinity purified antibodies or control IgG for 30 min at room temperature before addition of 60 \( \mu l \) of a 5% (v/v) suspension of inactivated Staphylococcus aureus strain Cowan 1 (Pansorbin, Calbiochem-Behring). After 30 min at 0 °C, the bacterial pellet was harvested by centrifugation and washed twice with 10 mM sodium phosphate, 1 mM EDTA, 0.4 M NaCl, 0.1% Triton X-100, pH 7.5. In the final wash the NaCl concentration was reduced to 0.1 M. The bacterial pellet was resuspended in 200 \( \mu l \) of 50 mM Tris-HCl, 0.1 mM inactivated Staphylococcus aureus of strain Cowan 1 (Pansorbin, harvested by centrifugation and washed twice with 10 mM sodium phosphate). After 30 min at 0 °C, the bacterial pellet was removed by centrifugation, the radioactivity remaining in solution was determined. B, unlabelled purified inhibitor-2, as indicated in the figure, was mixed with \( ^{32}P \)-labelled inhibitor before exposure to a fixed amount (2 \( \mu l \)) of immune serum. Increasing \( ^{32}P \) in solution then represents competition by the unlabelled inhibitor-2 for antibody binding.

**Results**

**Characterization of Antibodies**—Initial characterization of the immune serum utilized inhibitor-2 that had been labeled with \( ^{32}P \) after phosphorylation by casein kinase II (see Ref. 12). Immunoprecipitation was carried out by harvesting antigen-antibody complexes with \( S. \) aureus. As seen in Fig. 1, almost complete removal of inhibitor-2 (70 ng) from solution was achieved with 2 \( \mu l \) of immune serum. No inhibitor-2 was precipitated with the control serum. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis in the presence of SDS indicated that all the radioactivity removed was associated with inhibitor-2 (not shown). Also, addition of unphosphorylated inhibitor-2 to the \( ^{32}P \)-labeled inhibitor before exposure to antibodies reduced the amount of \( ^{32}P \) immunoprecipitated (Fig. 1), indicating competition for the antibodies. The results demonstrate first the ability of the antibodies to recognize purified inhibitor-2. Secondly, the antibodies do not distinguish between unphosphorylated inhibitor-2 and inhibitor that was phosphorylated by casein kinase II.

**Analysis of Inhibitor-2 by Immunoblotting**—Extracts were prepared from various rabbit tissues, as described under "Experimental Procedures," either with or without heat treatment of the extracts. These samples, together with a standard of purified rabbit muscle inhibitor-2, were subjected to polyacrylamide gel electrophoresis in the presence of SDS, transferred to nitrocellulose, and probed with antibodies against inhibitor-2. In Fig. 2 are shown the results of such an experiment using affinity purified antibodies. The corresponding control, using nonimmune IgG as a probe, had no radioactive species detectable on the autoradiogram (not shown). With unboiled extracts, several immunoreactive species were found for each of the tissues analyzed. The patterns were to some degree tissue specific. Prominent species with apparent \( M, \) approximately 39,000, were seen in all of the tissues except lung. In skeletal muscle, diaphragm, and liver, another polypeptide of apparent \( M, \) 58,000 was visible and a species of apparent \( M, \) 50,000 was common to liver, kidney, lung, and perhaps brain. Brain was characterized by a strongly interacting species of \( M, \) 60,000–66,000. A few very high molecular weight polypeptides were also apparent. Of interest are species of approximately 115,000 in skeletal and diaphragm muscle, and of 136,000 in liver, kidney, and lung. Some other species can be seen in the figure. Relatively less prominent but still clearly detectable in all tissues, however, was a species with the electrophoretic mobility of inhibitor-2, apparent \( M, \) 31,000.

The parallel analysis of boiled extracts indicated much simpler patterns. The species of \( M, \) 31,000, corresponding to inhibitor-2, was now prominent in all tissues (Fig. 2) though present perhaps in lower amount than in unboiled extracts. The dominant brain polypeptide(s) of 60,000 to 66,000 daltons remained. The only other species detected were the very high molecular weight ones, 115,000 for skeletal and diaphragm muscle and 136,000 for liver, kidney, and lung.

The basic features of the results just described were not altered by a number of variations in the procedures both

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\(^{2}\) A. A. DePaoli-Roach, unpublished results.
related to the Western blotting (see "Experimental Procedures") and in sample preparation. Omission of protease inhibitors and EGTA from the homogenization buffer did modify the patterns slightly where tested (with liver, skeletal muscle, and diaphragm) but did not lead to any increase in the amount of the 31,000-dalton species corresponding to inhibitor-2. In fact, in the boiled liver extract, the amount of inhibitor-2 was clearly decreased if the protease inhibitors were omitted, with the appearance of smaller immunoreactive polypeptides (not shown). Initially, immune serum was used instead of purified antibodies. No major differences in the results were found except that a prominent species was detected, at around 60,000, in all the tissue extracts. Presumably, this species was not due to antibodies that bound inhibitor-2 during affinity purification.

Immunoprecipitation of Inhibitor-2 from Liver and Muscle—Further experiments were carried out using immunoprecipitation which allowed physical separation of the material recognized by the antibodies. After exposure of extracts, boiled or unboiled, to antibodies, the antibodies were harvested with S. aureus, as described under "Experimental Procedures." Material released from the bacterial pellets by boiling was analyzed by gel electrophoresis followed by immunoblotting (Fig. 3) or assayed for phosphatase inhibitory activity (Table I). The effective detection of purified inhibitor-2 (66 ng) by immunoblotting is indicated by track 1 (Fig. 3). From Fig. 3, it is apparent that the main species immunoprecipitated from either liver or muscle extracts had apparent M, 31,000. No such species was present when nonimmune IgG were used in the immunoprecipitation. Similar results were obtained using material released from the bacterial pellet by 70% formic acid rather than boiling (data not shown). Traces of the very high M, polypeptides were also present. It should be noted that the relative abundance of these low mobility polypeptides was somewhat variable in these experiments. In addition, comparison of the results using boiled or unboiled extracts indicated that the yield of 31,000-dalton polypeptide was not greatly affected by heat treatment of the tissue extracts. Table I indicates that the material released from the immunoprecipitates by boiling, from liver or muscle extracts, was able to inhibit purified type 1 protein phosphatase. Therefore, the evidence strongly argues that the species of apparent M, 31,000 visualized in this study indeed corresponds to inhibitor-2.

FIG. 2. Immunoblotting of extracts of various rabbit tissues. For all three panels, extracts (10 μl) of the following tissues were analyzed: track 1, skeletal muscle; track 2, diaphragm muscle; track 3, liver; track 4, heart; track 5, kidney; track 6, lung; and track 7, brain. Polyacrylamide gel electrophoresis in the presence of SDS, 6–20% acrylamide gradient gels, was used for analysis of unboiled tissue extracts (B and C) or boiled extracts (A). Transfer to nitrocellulose, incubation with antibodies, and visualization of antibodies with protein A were as described under "Experimental Procedures." Panel C, nitrocellulose filter stained with Amido Black after transfer of samples from unboiled extracts to show the multiplicity of proteins present on the filter. Panel B, autoradiogram (15-h exposure) corresponding to panel C indicating immunoreactive species. Track 8 contained 66 ng of purified inhibitor as a standard. Panel A, autoradiogram (48-h exposure) of nitrocellulose filter to which samples from boiled extracts were transferred. Essentially no protein was visible by staining of the filter. For all the figure, the two-digit numbers are the apparent molecular weights (× 10^3). Note that there are slight scale differences in the different panels.

FIG. 3. Analysis of immunoprecipitates from rabbit skeletal muscle and liver extracts by Western blotting. Immunoprecipitates prepared from either native (tracks 7–10) or boiled (tracks 3–6) extracts of muscle and liver were analyzed by immunoblotting, as described under "Experimental Procedures." An autoradiogram of the nitrocellulose filter is shown. Track 1, 66 ng of purified inhibitor-2 as a standard; track 2, a protein A control in which neither extract nor antibodies were included; tracks 3 and 4, boiled liver extracts immunoprecipitated with immune or control IgG, respectively; tracks 5 and 6, boiled muscle extracts immunoprecipitated with immune or control IgG; tracks 7 and 8, unboiled liver extracts immunoprecipitated with immune or control IgG; tracks 9 and 10, unboiled muscle extracts immunoprecipitated with immune or control IgG. For tracks 3–10, the equivalent to 44 μl of the material released from the immunoprecipitates was applied to the polyacrylamide gel. The two-digit numbers are apparent molecular weights (× 10^3) calculated from the migration of standard proteins.

DISCUSSION

The main experimental result of this investigation is straightforward. Inhibitor-2 can be detected in rapidly processed tissue extracts as a species of apparent M, 31,000. This species is the dominant one recognized by antibodies to inhibi-
**Table I**

*Phosphatase inhibitory activity associated with immunoprecipitates of rabbit muscle and liver extracts*

| Tissue   | Condition        | Activity of phosphatase type 1 | % of control |
|----------|------------------|--------------------------------|--------------|
|          |                  | Unboiled extract | Boiled extract |
| Skeletal | Control IgG      | 95                | 98           |
|          | Immune IgG       | 9                 | 11           |
| Liver    | Control IgG      | 105               | 97           |
|          | Immune IgG       | 21                | 17           |

The significance of the higher $M_s$ species detected by immunoblotting of unboiled extracts is not clear. From the immunoprecipitation experiments, either most of these species were not recognized in their underdenatured states by the antibodies or else they were not released by boiling or formic acid extraction of the immunoprecipitates. Since formic acid would be expected to solubilize most proteins, one possibility is that antigenic determinants common to inhibitor-2 were exposed only upon denaturation. Of course, an interesting question is whether such species have any functional and/or structural relationship to inhibitor-2. More detailed studies will be required but we can note that our experiments never indicated the generation of inhibitor-2 as a 31,000-dalton species. Boiling of extracts did not lead to the accumulation of inhibitor-2 nor did omission of protease inhibitors from the homogenization buffer. These results are not definitive but do not provide any strong indications that inhibitor-2 is produced by manipulation of the tissue extracts.

The most important conclusion of the study relates to the tissue distribution of inhibitor-2 in a form consistent with our knowledge of the muscle inhibitor. As noted in the introduction, relatively little structural information is available concerning inhibitor-2 in non-muscle tissues. From the results, it is evident that rabbit liver contains an antigenically related species of similar molecular weight. In liver extracts, this protein was more susceptible to proteolytic degradation, which could correlate with the identification of a lower molecular weight form of phosphatase inhibitor in liver in an earlier study (15). From immunoblotting of boiled extracts of other rabbit tissues (cardiac and diaphragm muscle, kidney, brain, and lung) we can infer that inhibitor-2 is indeed a widely distributed protein. The function of inhibitor-2 in the control of phosphatase activity is therefore likely to be important for protein dephosphorylation in many, if not all, mammalian tissues.

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