A Biologically Active Form of Chromium May Activate a Membrane Phosphotyrosine Phosphatase (PTP)†

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ABSTRACT: Chromium is essential for proper carbohydrate and lipid metabolism in mammals, although the mechanism of this action has previously proved elusive. Low-molecular-weight chromium-binding protein (LMWCr), a biologically active form of chromium in mammals, potentiates the effect of insulin on the conversion of glucose into lipid and into carbon dioxide in isolated adipocytes. Kinetics studies indicate that LMWCr isolated from bovine liver activates phosphotyrosine phosphatase (PTP) activity in adipocyte membranes while having no intrinsic phosphatase activity. This activation is directly proportional to the amount of added LMWCr. The pattern of inhibition of this activity in the presence of a number of known phosphatase inhibitors suggests the involvement of a membrane phosphotyrosine phosphatase similar to PTP1A′ or PTP1B. We propose that chromium plays a biological role in the activation of a membrane phosphotyrosine phosphatase.

The first-row transition elements from vanadium to zinc are each essential for some form of life (Kaim & Schwederski, 1994; Lippard & Berg, 1994; Frausto da Silva & Williams, 1991). For each metal except chromium, at least one (and usually many) metallobiomolecule containing an ion of that element has been well-characterized in terms of its function and mode of action. Since its inception nearly forty years ago (Schwarz & Mertz, 1959), chromium biochemistry has proved to be an enigma (Vincent, 1994a). In the mid 1950's, rats fed a *Torula* yeast diet [which proved to be Cr-deficient (Anderson, et al., 1978)] developed glucose intolerance which could be reversed only by addition of Cr to the diet (Schwarz & Mertz, 1957, 1959; Mertz & Schwarz, 1959). A Cr-rich material extracted from Brewer's yeast, named glucose tolerance factor (GTF),[1] was found to be especially effective in reversing the glucose intolerance (Schwarz & Mertz, 1959). Unfortunately, the isolation of this material involved procedures such as an 18-h reflux in 5 N HCl which would have destroyed any protein or similar components (Toepfer et al., 1977); the isolation and characterization of this material have not proved to be reproducible in some laboratories (Haylock et al., 1983; Gonzalez-Vergara et al., 1982; Shepherd et al., 1992). Additionally, recent analysis of kinetics studies on the biological activity of GTF indicates that it serves only as a readily absorbable form of Cr and that this yeast material has no intrinsic function in mammals (in fact, it may inhibit insulin in non-Cr-deficient cells) (Vincent, 1994b). [Biological activity is defined as the ability to potentiate the effects of insulin on conversion of glucose into carbon dioxide or into lipid by epididymal fat tissue or isolated adipocytes (Mertz & Roginski, 1963; Anderson et al., 1978).] Recent interest has also been directed to chromium picolinates (Evans & Bowman, 1992; Hasten et al., 1992; Evans & Pouchnik, 1993); however, these materials also appear to serve only as readily absorbable sources of Cr (McCarty, 1993). Also because of the picolinate ligands, they cause chromosome damage (Stearns et al., 1995).

Nevertheless, medical studies clearly indicate that Cr is required for normal carbohydrate and lipid metabolism (Anderson, 1985, 1986). Cr deficiency in humans and other mammals results in symptoms comparable to those associated with adult-onset diabetes and cardiovascular disease: decreased glucose tolerance (Mertz et al., 1965; Woolliscroft & Barbosa, 1977; Hopkins et al., 1968; Schroeder, 1965), increased concentration of circulating insulin (Schroeder & Balassa, 1965), decreased insulin receptor number (Anderson et al., 1987), elevated cholesterol and triglyceride levels (Riales & Albrink, 1981), and reduced high-density-lipoprotein (HDL) cholesterol levels (Riales & Albrink, 1981; Anderson, 1987). Improvement in glucose tolerance after supplementation of the diet with chromium has been documented many times (Schwartz & Mertz, 1959; Anderson et al., 1983, 1987, 1991a; Glinsman & Mertz, 1966; Levine et al., 1968; Hopkins, 1965; Gurson & Saner, 1971; Martinez et al., 1985; Mahdi & Naismith, 1991), as well as improvements in other symptoms (Anderson, 1985, 1986; Riales & Albrink, 1981; Madhi & Naismith, 1991). Cr is additionally released from storage pools and resultantly excreted in the urine in response to certain stresses including high sugar intake (Martinez et al., 1985; Anderson et al., 1982). In particular, carbohydrates that alter circulating insulin levels also effect urinary Cr losses, as glucose tolerance decreases, the mobilization of Cr and resulting Cr loss have also been shown to decrease (Anderson et al., 1990). Yet as much as 90% of the American population and half of the population of developed nations daily intakes are less than the recommended safe and adequate quantities of Cr (Anderson & Kozlovsky, 1985; Anderson, 1994).

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[1] Abbreviations: GTF, glucose tolerance factor; LMWCr, low-molecular-weight chromium-binding substance; PTP, phosphotyrosine phosphatase; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; *p*-NPP, *para*-nitrophenyl phosphate; PP1, phosphoprotein phosphatase 1; PP2A, phosphoprotein phosphatase 2A; LAR, leukocyte antigen related phosphotyrosine protein phosphatase.

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At present, the only viable candidate for the biologically active form of Cr is low-molecular-weight Cr-binding substance (LMWCr) (Yamamoto et al., 1987; Davis and Vincent, submitted for publication). LMWCr is a mammalian polypeptide of circa 1500 Da which binds four chromic ions in a multinuclear assembly; this laboratory has recently developed a procedure for isolating milligram quantities of this polypeptide (Davis and Vincent, submitted for publication). Kinetics studies on LMWCr indicate that it has an intrinsic postreceptor role (i.e., after the binding of insulin to the external surface of insulin receptor) in the insulin-dependent activation of conversion of glucose into carbon dioxide and lipid (Vincent, 1994b; Yamamoto et al., 1988) and that this activation is directly dependent on the Cr content of LMWCr (Yamamoto et al., 1989). In addition, LMWCr is a major form of chromium in urine and bile (Manzo et al., 1983; Wada et al., 1983) and likely represents the form of Cr released in response to stress, such as carbohydrate stress. Because Cr(III) complexes are substitutionally inert, it is unlikely that LMWCr has a direct enzymatic function (Vincent, 1994a). The fact that insulin functions by activating a series of cascade systems involving multiple phosphorylation/dephosphorylation events (Saltiel, 1994) suggests that LMWCr may be involved in the regulation of these phosphorylation steps. Herein are reported kinetics studies which indicate that LMWCr activates a membrane PTP (phosphotyrosine phosphatase).

MATERIALS AND METHODS

LMWCr and ApoLMWCr. LMWCr was available from previous work (Davis and Vincent, submitted for publication). ApoLMWCr was prepared by heating a solution of LMWCr in 3.5 mM EDTA and a catalytic amount of sodium cyanoborohydride adjusted to pH 3 at 60 °C for 12 h. The apoprotein was separated from the EDTA and reductant by passing over a column of G-15 Sephadex. ApoLMWCr contains approximately 0.3 Cr per polypeptide. Chromium was assayed using the diphenylcarbizide procedure (Marczenko, 1986) utilizing the method of standard addition to minimize any potential matrix effects. Oligopeptide concentrations were assayed by the fluorescamine procedure of Undenfriend and co-workers (1972). The concentration of holoLMWCr in certain experiments is given in terms of chromium concentration as LMWCr; preparations of LMWCr in this work contained 3.6 chromium per oligopeptide.

Preparation of Isolated Adipocytes. Fat cells from male Sprague-Dawley rats were isolated by modifications of the procedure of Rodbell (1964). Three rats (not kept on a Cr-deficient diet) were sacrificed by decapitation, and their epididymal fat pads were removed. Subsequent operations followed Anderson et al. (1978) with minor modification, and 2% bovine serum albumin (BSA) media was changed to 1% bovine serum albumin and solutions were not gassed with O_2.

Adipocyte Membrane and Cytosol Preparation. Rat adipocytes were isolated as above except they were washed with 1% bovine serum albumin, 50 mM Hepes, pH 7.4 buffer containing 10 μg of leupeptin/mL and 5 μg of aprotinin/L. Cells were homogenized with a manual Teflon homogenizer and frozen and thawed five times. The lipid layer was removed, and the cell homogenate was centrifuged for 1 h at 40 000*g*. The supernatant was removed and used as a source of cytosol phosphatase activity. The pellet was suspended in Hepes buffer and used as a source of membrane phosphatase activity. Protein concentrations were determined using the BCA method (Pierce Chemical Co.) with BSA as standard.

Phosphotyrosine Phosphatase and Phosphoserine/Phosphothreonine Phosphatase Activity. PTP activity was estimated using *p*-nitrophenyl phosphate (*p*-NPP) by the method of Li et al. (1995). The assay used 5 mM substrate in 0.05 M Tris, pH 7.5, unless otherwise noted. Hydrolyses were allowed to proceed 1 h at 37 °C. The extent of hydrolysis was determined at 404 nm ($\epsilon = 1.78 \times 10^4\ M^{-1}\ cm^{-1}$). PTP activity was also determined using a phosphotyrosine assay kit (Boehringer Mannheim), which uses a fragment of human gastrin (amino acids 1−17) phosphorylated on tyrosine-12 and a synthetic fragment of hirudin (53−65 C-terminal fragment) phosphorylated on tyrosine-63 as substrates. These assays were also performed in 0.05 M Tris, pH 7.5, and hydrolysis reactions were allowed to proceed 1 h at 37 °C unless otherwise noted. When the kit was used, membranes (which contain phosphotyrosine themselves) were removed after the reaction using Microcon 30 microconcentrators (Amicon); 0.8 mM piceatannol was used as a kinase inhibitor. Bovine intestinal mucosa alkaline phosphatase (Sigma), *Yersinia* PTP fragment (Boehringer Mannheim), catalytic subunit of the γ form of human protein phosphatase 1 (PP1) (Boehringer Mannheim), human transmembrane leukocyte antigen related tyrosine phosphatase (LAR) (Calbiochem), $[NH_4][O_2CMe]$, LMWCr, and apoLMWCr were assayed for PTP activity using 5 mM *p*-NPP in 0.05 M Tris, pH 7.5, at 37 °C for 1 h except the reaction with alkaline phosphatase which was allowed to proceed 15 s at room temperature, the reaction with LAR (10 units) which was allowed to proceed for 45 min at 37 °C, and the reaction with PP1 (0.05 milliunits) which was allowed to proceed for 2 h at 37 °C. For experiments utilizing monoclonal antibodies whose epitope is within the catalytic domain of human PTP 1B (and which reacts with rat PTP 1B) (Calbiochem), PTP activity of rat adipocyte membranes was assayed as above except membranes (corresponding to 5 μg of protein) were incubated for 2 h at 4 °C with 1.09 μg of antibody before addition of *p*-NPP; the hydrolysis reactions were allowed to proceed 75 min at 37 °C. Phosphoserine/phosphothreonine phosphatase activity was measured using 0.83 mM phosphvitin as substrate in 0.05 M Tris, pH 7.5, at 37 °C by following phosphate release after 1 h by the method of Zhang and VanEtten (1991). Curve-fitting was performed using SigmaPlot (Jandel Scientific).

Inhibitor and Metal Reconstitution Studies. Metals were used as the following salts: $NiSO_4 \cdot 6H_2O$ (J. T. Baker), $Zn(NO_3)_2 \cdot 6H_2O$ (Mallinckrodt), $Cu(SO_4) \cdot 5H_2O$ (Fisher), $CoCl_2$ (J. T. Baker), $[NH_4]VO_3$ (Fisher), $FeCl_3 \cdot 6H_2O$ (J. T. Baker), $Cr(NO_3)_3 \cdot 6H_2O$ (Fisher), $CrCl_3 \cdot 6H_2O$ (Fisher), $MnSO_4 \cdot H_2O$ (Mallinckrodt), and $Na_2MoO_4 \cdot 2H_2O$ (Fisher). Metals and inhibitors were added to the reaction mixture and incubated for 10 min at 37 °C prior to reaction initiation by addition of substrate. Microcystin LR was obtained from Calbiochem.

Miscellaneous. All visible spectroscopic measurements were made with a Hewlett-Packard 8451A diode array spectrophotometer. EPR were collected on a Varian E-12 spectrophotometer equipped with an Oxford ESR 900

cryostat. For apoLMWCr, solutions in 0.050 M NH_4OAc were rapidly frozen in liquid N_2. Integrations were performed as previously described using hexaaquochromium-(III) as a standard (Davis and Vincent, submitted for publication); the broad EPR signal of pseudooctahedral mononuclear Cr^{3+} requires the use of a standard with a similarly broad EPR signal for accurate integrations. Aqueous solutions of $[Cr(H_2O)_6]^{3+}$ were rapidly frozen in liquid N_2; the integrity of the sample was checked by UV/visible spectroscopy after the sample was thawed at the conclusion of the EPR experiment. Fluorescence measurements were obtained with a Perkin Elmer 204 fluorescence spectrophotometer. All kinetics experiments were performed in triplicate and reproduced at least once. Errors are presented throughout including all tables and graphs as the standard deviations (1σ) of the triplicate analyses. Similarly, all Cr and LMWCr concentration determinations were made in triplicate. Doubly deionized water was used in all operations.

RESULTS AND DISCUSSION

Phosphatase Activation

LMWCr functions in a manner in insulin-sensitive cells such that the action of insulin is potentiated. In insulin dose−response studies, the degree of incorporation of ^{14}C or ^{3}H from labeled glucose into carbon dioxide or total lipids is enhanced while the concentration of insulin for half-maximal activity is unaffected (Yamamoto et al., 1988; Davis and Vincent, submitted for publication). This indicates that LMWCr has an intrinsic role "inside the cell" which is stimulated by the action of insulin while not affecting the interaction of insulin with its receptor. Given the unlikely possibility that LMWCr acts as a catalyst and that insulin action is propagated by series of phosphorylation/dephosphorylation events, a role for LMWCr in the activation or inhibition of kinases or phosphatases associated with insulin action seemed plausible. Therefore, the effects of LMWCr on rat adipocyte phosphatases were examined. Rat adipocytes were homogenized and separated into three portions: lipids, soluble, and membrane/particulate. LMWCr had no effect on phosphatase activity toward *p*-NPP in either the lipid or soluble portions (not shown); however, a distinct effect was observed in the phosphatase activity associated with the membrane fragments (Figure 1). The ammonium acetate buffer in which LMWCr is stored displayed no ability to activate the phosphatase activity, and LMWCr in the absence of the membranes also demonstrated no catalytic activity. Over the concentration range 0.1 nM to 10 μM (in terms of Cr), the addition of LMWCr activates the arylphosphate phosphatase activity of the membranes in a concentration dependent fashion. Fitting the hyperbolic phosphatase activation behavior to the Henri−Michaelis−Menten equation yields a LMWCr dissociation constant of 4.4 nM. Similar results over the same Cr concentration range have also been obtained utilizing adipocytes isolated from adipose tissue trimmed from bovine liver. To determine whether this arylphosphate phosphatase activity corresponded to phosphotyrosine phosphatase activity, the ability of LMWCr to activate the hydrolysis of phosphotyrosine-containing polypeptides was examined. Using human gastrin (amino acids 1−17) phosphorylated on tyrosine-12 and a synthetic fragment of hirudin (53−65 C-terminal fragment) phosphorylated on tyrosine-63 as substrates and the adipocyte membrane

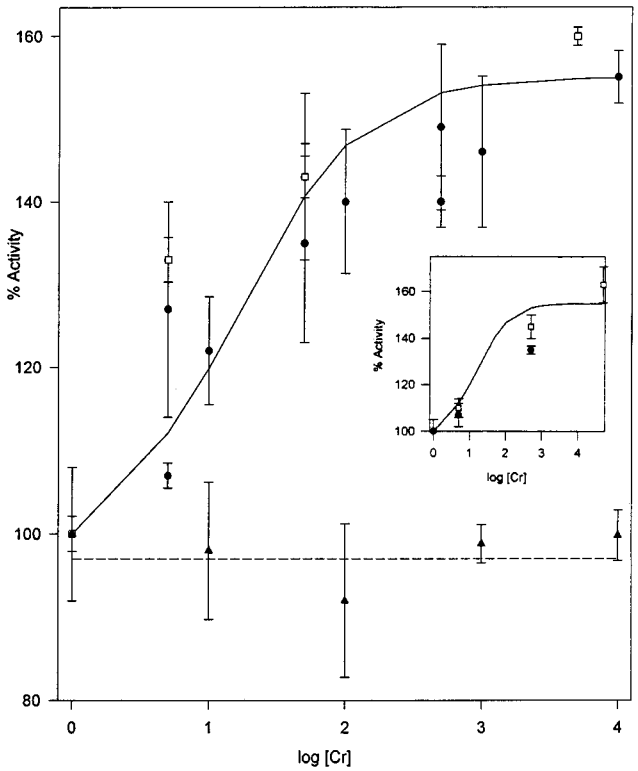

FIGURE 1: Activation of rat adipocytic membrane phosphotyrosine phosphatase activity using 5 mM *p*-NPP as substrate by LMWCr (solid circles) and rat adipocyte membrane phosphoserine phosphatase activity using 0.83 mM phosvitin as substrate (shaded triangles). 125 μL of a rat membrane suspension corresponding to 136.8 μg of protein/mL was utilized. Activation of phosphotyrosine phosphatase activity of *Yersinia* phosphotyrosine phosphatase fragment (19.5 milliunits) using 5 mM *p*-NPP as substrate by LMWCr (open squares). The line is the best fit hyperbolic curve giving a LMWCr dissociation constant of 4.4 nM. Inset: Activation of rat adipocyte membrane phosphotyrosine activity using 0.75 μM human gastrin fragment (open squares) or hirudin fragment (solid circles) as substrate. 125 μL of a rat membrane suspension corresponding to 129 μg of protein/mL was utilized. The line is the same best fit curve as above. All Cr concentrations are presented in units of nM.

fragments, LMWCr activated hydrolysis in a concentration dependent manner (Figure 1), nearly identical to that of *p*-NPP by the membrane fragments. For example, 72% activation of hirudin dephosphorylation was observed at an LMWCr concentration corresponding to 50 μM chromium; 63% activation of gastrin dephosphorylation was observed at the same LMWCr concentration. These phosphotyrosine-containing peptide assays were performed in the presence of the kinase inhibitor piceatannol, suggesting that phosphatase activation and not kinase inactivation is responsible for this activity. When the phosphoserine-containing protein phosvitin was used as a substrate, no activation of dephosphorylation this protein was observed over the same range of LMWCr concentrations (Figure 1). The activation potential of LMWCR is therefore likely to be directed toward phosphotyrosine phosphatase activity.

Inhibition Studies

Rat adipocyte membranes possess a number of phosphatases including phosphoserine/phosphothreonine phosphatases and phosphotyrosine phosphatases (Ding et al., 1994; Liao et al., 1991; Begum, 1995). PP1 comprises 80% of phosphoserine/phosphothreonine phosphatase activity in

Table 1: Inhibition of Membrane PTP Activity and LMWCr-Activated Membrane PTP Activity Using 5 mM *p*-NPP as Substrate[a]

inhibitor	% activity (− LMWCr)	% activity (+ LMWCr)
none	100(4)	191(8)[c]
EDTA (5 mM)	148(10)	149(5)
microcystin LR (1 μM)	110(3)	119(4)[c]
spermine (2 mM)	115(5)	126(5)[d]
spermidine (2 mM)	109(2)	122(2)[c]
zinc nitrate (100 μM)	77(1)	86(2)[c]
apoLMWCr (12.5 μM)	133(2)	−
apoLMWCr (12.5 μM) + $CrCl_3$ (50 μM)	197(7)[e]	−
$CrCl_3$ (50 μM)	102(10)	−

[a] Standard deviations are given in parentheses. [b] 50 μM Cr. [c] Differs significantly ($P < 0.002$) from the value of the assay without LMWCr (Student's unpaired *t*-test). [d] Differs significantly ($P < 0.01$) from the value of the assay without LMWCr. [e] Differs significantly ($P < 0.0002$) from the value of the assay without $CrCl_3$.

rat adipocyte membranes, while PP2A is apparently restricted to the cytosol (Begum, 1995); rat adipocytes contain virtually no PP2B activity and only low levels of PP2C activity (Wood et al., 1993). To determine which type of phosphatase was activated by LMWCr, the effects of the addition of specific phosphatase inhibitors on the activation by LMWCr were examined, as was the effect of LMWCr on isolated phosphatases. In the same concentration range used with the membrane fragments, LMWCr had no effect on alkaline phosphatase or phosphoprotein phosphatase 1 (PP1). In contrast, LMWCr activates the hydrolysis of *p*-NPP by the catalytic fragment of *Yersinia* phosphotyrosine phosphatase; the concentration dependence of the activation of the fragment is essentially identical to that of the membrane fragments (Figure 1), indicating that LMWCr must be activating a PTP. The use of the isolated phosphatase clearly suggests that inactivation of a kinase is not responsible for the observed apparent dephosphorylation.

Rat adipocytes contain a number of PTP's including LRP/RPTPα, PTP 1B, SH-PTP2/Syp, LAR (Ding et al., 1994), HA1 and HA2 (Liao et al., 1991). [HA2, however, is a homolog of PTP 1B (Liao & Lane, 1995).] LAR, LRP/RPTPa, HA1, and HA2 are membrane proteins. PTP's can be broadly distinguished by their behavior in the presence of a variety of inhibitors (Pot & Dixon, 1992). For example rat LAR is inhibited by EDTA, spermine, and spermidine and almost unaffected by zinc cations (Pot et al., 1991); the human analogue of RPTPα is inhibited by zinc, EDTA, and spermine (Wang & Pullen, 1991). In contrast, rat adipocyte membrane phosphotyrosine phosphatase activity demonstrates a different pattern of inhibition (Table 1). The PTP activity is activated by EDTA, spermine, and spermidine but inhibited by zinc cations. The activity is not consistent with the dominate phosphatase activity being LAR, RPTPa, or even alkaline phosphatase (inhibited by EDTA as it possesses two zinc ions at its active site) (Vincent & Crowder, 1995). Similarly, microcystin LR, a specific inhibitor of PP1 and PP2A at nanomolar concentrations (Honkanen et al., 1990), does not inhibit overall phosphatase activity. However, microcystin does inhibit the activation of phosphatase activity by LMWCr; the origin of this effect is uncertain. The inhibition/activation pattern, however, corresponds to that of two types of membrane PTP's isolated from human placenta (Tonks et al., 1988c), P1A′ and P1B. P1B, for example, is activated toward hydrolysis of phosphotyrosyl RCM lysozyme 166% by 5 mM EDTA, 40% by 2 mM spermine, and 138% by 2 mM spermidine and is inhibited 94% by 100 μM Zn^{2+}. The striking similarity between the P1A′ and P1B enzymes and the major PTP of the adipocyte membranes suggests that they are probably related. HA1 and HA2 are completely inhibited by 1 mM Zn^{2+} (Liao et al., 1991). HA2, which is a PTP 1B homolog, possesses approximately the same molecular weight as isolated P1B (Liao et al., 1991; Tonks et al., 1988b, 1991) and may, then, represent the major PTP activity in the membrane fragments. The activation of the membrane PTP activity by LMWCr is inhibited by the addition of the other activators (Table 1). This suggests that LMWCr activates HA2 or a PTP with similar behavior toward EDTA and the polycationic compounds. LMWCr could activate other PTP's, but their contribution cannot be elucidated by these studies. Curiously, microinjection of soluble placental PTP 1B into *Xenopus* oocytes is antagonistic to insulin action (Cicirelli et al., 1990); however, the enzyme seems to associate with internal membrane fragments (rather than the plasma membrane) where it could block specific physiological responses to insulin (Tonks et al., 1988a). However, insulin results in the stimulation of the phosphatase activity of SH2-PTP2 (also known as syp, PTP2C, or PTP1D) and SH2-PTP1 (also known as PTP1C, HCP, or SHP) (Sugimoto et al., 1994; Uchida et al., 1994). It is conceivable that the ability of the reagents in Table 1 to inhibit activation by LMWCr of PTP activity could be by interaction with LMWCr (making conclusions about the identity of the PTP difficult); however, as each of these reagents is an inhibitor or activator of the PTP activity in the absence of LMWCr and, thus, known to bind to the PTP already, this possibility is unlikely.

Studies of the activation of the membrane PTP by LMWCr as a function of LMWCr and substrate concentration reveal that not all of the phosphatase activity associated with the membranes is activated by LMWCr (Figure S1 in supporting information), as indicated by the curvatures of the lines in the Lineweaver−Burk plot, distinctive of curvilinear (complex) activation. This is to be expected as the membranes certainly must contain PP1 and other phosphatases, capable of dephosphorylating *p*-NPP. Differential activation of the various membrane PTP's such as LAR, RPTPα, etc. may also add to the curvature. As the curve is linear in the absence of LMWCr, an apparent K_m of 632 μM can be estimated for the overall hydrolysis. This value is similar to the K_m reported for *p*-NPP hydrolysis catalyzed by PTP's [for example for rat LAR, $K_m = 420$ μM (Pot et al., 1991)]. Thus, the major component of the rat adipocyte membrane PTP activity is affected by addition of LMWCr and has a pattern of inhibition and activation by certain reagents similar to that of PTP1A′ and PTP2B; this suggests the that activation of PTP activity by LMWCr involves of a membrane PTP similar to PTP1A′ or PTP2B.

Incubation of rat adipocyte membranes with monoclonal antibodies whose epitope is the catalytic domain of human PTP1B and which react with rat PTP1B results in the reduction of the ability of LMWCr to stimulate PTP activity; when 50 μM Cr is used as LMWCR, the antibodies result in a loss of 78% of the oligopeptide's ability to potentiate PTP activity. Hence, the PTP(s) activated by LMWCr must be PTP1B and/or closely related phosphotyrosine protein phosphatases. This is also supported by studies using the

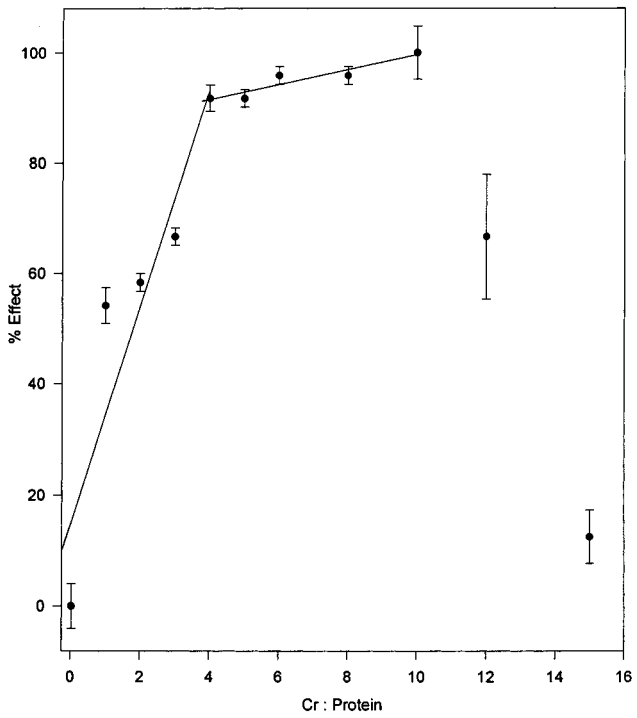

FIGURE 2: Chromic ion titration of the ability of apoLMWCr to activate the hydrolysis of *p*-NPP by rat adipocyte membrane fragments. The concentration of apoLMWCr was 12.5 μM. 125 μL of a rat membrane suspension corresponding to 326 μg of protein/mL was utilized.

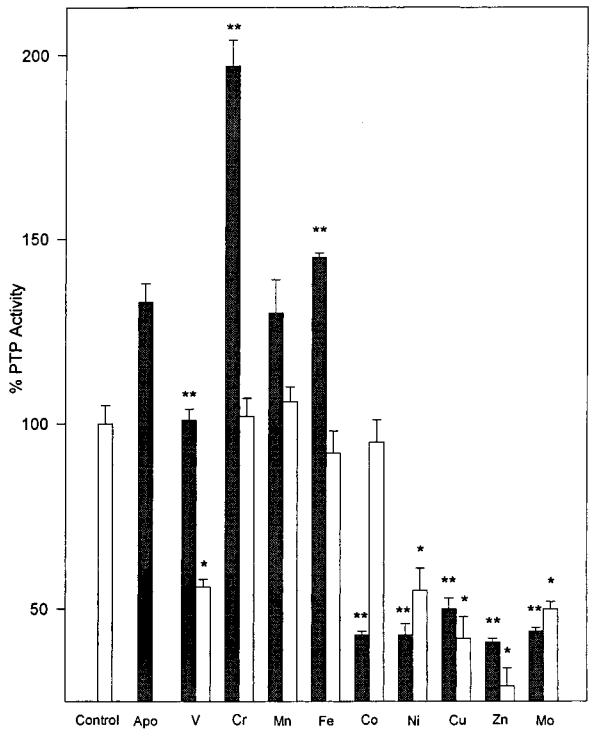

FIGURE 3: Activation of rat adipocyte membrane phosphotyrosine phosphatase activity using 5 mM *p*-NPP as substrate by apoLMWCr (25 μM) and metal ions (100 μM). Bars marked with "*" differ significantly ($P < 0.0002$) from the control assays (i.e., in the absence of added metal) (unpaired Student *t*-test), and those marked with "**" differ significantly ($P < 0.0002$) from the apoLMWCr assays in the absence of added metal.

isolated phosphotyrosine protein phosphatase LAR, which has distinct inhibition properties from PTP1A′ and PTP1B; no activation of this enzyme's activity towards the dephosphorylation of *p*-NPP was observed over a range of LMWCr concentrations (Cr concentrations varying from 5 nM to 50 μM).

Metal Reconstitution

Apoprotein (which contains circa 0.3 Cr) activates PTP activity slightly (~33%), while addition of apoprotein and four equivalents of chromic ions completely restores the activation potential of the polypeptide (Table 1). The activation by high concentrations of apoprotein suggests that only a tiny fraction of the protein still binding chromium is present in the form containing four Cr centers while the remainder probably contain inactive mononuclear chromium centers. Integration of EPR spectra of apoprotein [using hexaaquochromium(III) as standard] indicates that essentially all of the chromium exists as mononuclear centers. Titration of apoprotein with chromium(III) reveals that 3.89 Cr/protein are required for complete restoration of the phosphatase activation activity (Figure 2). This is consistent with reports that LMWCr isolated from mammalian liver possesses four chromic ions per polypeptide (Yamamoto et al., 1987). [However, addition of more than 10 equiv results in inhibition of the activation activity.] Chromic ions by themselves are ineffective. PTP activation is, thus, dependent on the Cr content; insulin potentiation by LMWCr is similarly dependent on the Cr content (Yamamoto et al., 1989), suggesting that the PTP activation is related to insulin potentiation. Hence, the metal would appear to be important in maintaining the proper conformation of the polypeptide. With the exception of ferric ions, transition metal ions commonly associated with biological systems (other than Cr) are ineffective in potentiating the ability of apoprotein to activate the PTP activity (Figure 3).

Manganous ions have no effect on apoprotein, while all others except ferric ions are inhibitory. Even activation by ferric ions is extremely small although significant statistically ($P < 0.0002$). LMWCr would appear then to be specific for Cr^{3+}. [In the absence of LMWCr, these transition metal ions with the exception of Co, Fe, and Mn display inhibition of phosphatase activity as in the presence of LMWCr. Co, Mn, and Fe had no effect on phosphatase activity in the absence of LMWCr (Figure 3). None of the metals examined in the absence of apoprotein resulted in activation of the phosphatase activity. The effect of the addition of cobalt to apo-oligopeptide is most interesting as it differs appreciably from the effect in the absence of apoLMWCr; this suggests cobalt may bind appreciably to LMWCr and that the resulting Co-LMWCr complex (although inactive) could serve as a new spectroscopic probe of LMWCr.] The ferric ions probably associate with the apoprotein because of their similar charge to size ratio to chromic ions, giving a conformation similar to that of the native polypeptide; note that *in vitro* chromic ions compete for ferric ions in iron-binding proteins such as transferrin (Aisen et al., 1969). How selectivity for chromium versus iron is achieved *in vivo* is currently under investigation.

CONCLUSIONS

If LMWCr activates a PTP in response to insulin, the question arises as to what aspect of insulin action results in "turning on" LMWCr to activate the PTP. A clue may come from the observation that within 90 min of ingestion of glucose by humans, plasma insulin levels increase with a

parallel increase in urinary chromium loss (Anderson et al., 1990); this suggests a mobilization of chromium in response to insulin. As a low-molecular-weight Cr-containing species may represent the form of Cr(III) in urine (Manzo et al., 1983), LMWCr may somehow be mobilized or apoLMWCr loaded with Cr in response to insulin. In order to address these issues, studies are in progress to examine the distribution of LMWCr in rat hepatocytes before and after treatment with insulin.

In conclusion, LMWCr activates a membrane phosphotyrosine phosphatase in a manner dependent on the Cr content of the oligopeptide. Indirect evidence suggests that this activation may play a role in the potentiation of insulin action by LMWCr. Establishing whether this activation activity has a direct role in insulin potentiation will require *in vivo* studies now in preparation. The previous assays for chromium biological activity all involved the use of ^{14}C- or ^{3}H-labeled substrates; this assay may provide a simple, nonradioactive alternative for determining this activity.

SUPPORTING INFORMATION AVAILABLE

Lineweaver−Burk plot of LMWCr concentration and *p*-NPP substrate concentration dependence of rat adipocyte membrane PTP activity (1 page). Ordering information is given on any current masthead page.

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