Chromium Oligopeptide Activates Insulin Receptor Tyrosine Kinase Activity†

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ABSTRACT: A possible new mechanism for the amplification of insulin receptor tyrosine kinase activity in response to insulin has been identified. The chromium-containing oligopeptide low molecular weight chromium-binding substance (LMWCr) does not effect the tyrosine protein kinase activity of rat adipocytic membrane fragments in the absence of insulin; however, insulin-stimulated kinase activity in the membrane fragments is increased up to 8-fold by the oligopeptide. Using isolated rat insulin receptor, LMWCr has been shown to bind to insulin-activated insulin receptor with a dissociation constant of circa 250 pM, resulting in the increase of its tyrosine protein kinase activity. The ability of LMWCr to stimulate insulin receptor tyrosine kinase activity is dependent on its chromium content. The results appear to explain the previously poorly understood relationship between chromium and adult-onset diabetes and cardiovascular disease.

Chromium has been known to be essential for proper carbohydrate and lipid metabolism in mammals for nearly forty years, yet the structure, function, and mode of action of the biologically-active chromium-containing species have been enigmas (Vincent, 1994a). Recently a candidate for the biologically active form of chromium, low molecular weight chromium-binding substance (LMWCr)[1] (Yamamoto et al., 1987, 1988), has been isolated in sufficient quantity to allow for the examination of any potential biological function of this naturally-occurring oligopeptide (Davis et al., 1996; Davis & Vincent, 1997). LMWCr is an oligopeptide with a molecular weight of approximately 1500 daltons which binds four chromic ions per molecule (Yamamoto et al., 1987; Davis & Vincent, 1997). More than half the amino acids which comprise the oligopeptide are glutamate and aspartate residues, which appear to be important in chromium ligation. Insulin dose dependence studies of glucose incorporation into carbon dioxide and lipid by isolated rat adipocytes in the presence of varying concentrations of LMWCr have indicated that LMWCr has a role in the potentiation of insulin action subsequent to the binding of insulin to the receptor but prior to metabolism of glucose to give carbon dioxide and lipid (Yamamoto et al., 1988; Vincent, 1994b). Given this and the kinetic inertness of Cr(III) complexes such that LMWCr is not expected to have any type of enzymatic function, a role for LMWCr in regulation of phosphorylation/dephosphorylation events was investigated. While LMWCr appears to activate a membrane phosphotyrosine phosphatase (Davis et al., 1996), herein are reported studies which suggest that LMWCr's primary function may lie in the activation of insulin receptor tyrosine protein kinase activity in response to insulin.

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[1] Abbreviations: LMWCr, low molecular weight chromium-binding substance; PTP, phosphotyrosine phosphatase; BSA, bovine serum albumin; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; *p*-NPP, *para*-nitrophenyl phosphate; IRS-1, insulin receptor substrate 1.

MATERIALS AND METHODS

LMWCr and ApoLMWCr. LMWCr and apoLMWCr were available from previous work (Davis et al., 1996; Davis & Vincent, 1997). Chromium was assayed using the diphenylcarbazide 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) with glycine as standard. The concentration of holoLMWCr in certain experiments is given in terms of chromium concentration as LMWCr; preparations of LMWCr in this work contained 3.6−4.0 chromium per oligopeptide. Solutions of LMWCr used in the kinetic assays were prepared by dilution of more concentrated stock solutions.

Purification of Isolated Adipocytes and Adipocytic Membranes. Fat cells from male Sprague-Dawley rats were isolated by modifications (Davis et al., 1996) of the procedures of Rodbell (1964) and Anderson et al. (1978). Rat adipocytes were washed with 1% bovine serum albumin, 50 mM Hepes, pH 7.4, buffer containing 10 μg/mL of leupeptin and 5 μg/mL of aprotinin. 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 40000*g*. The pellet was suspended in Hepes buffer and used as a source of membrane phosphatase and kinase activity. Protein concentrations were determined using the BCA method (Pierce Chemical Co.) with BSA as standard.

Tyrosine Protein Kinase Activity. Phosphotyrosine was assayed using a protein tyrosine assay kit (Boehringer Mannheim) which uses a anti-phosphotyrosine antibody to recognize phosphotyrosine. Fragments of cell division kinase $p34^{cdc2}$ (amino acids 6−20) and gastrin (amino acids 1−17) which have been biotinylated such that they may be immobilized to streptavidin-coated microtiter plates (Boehringer Mannheim) were used as substrates. The assays were performed in 50 mM Tris, pH 7.4, containing 0.75 μM ATP and 7.5 μM $MgCl_2$ at 37 °C for 75 min unless otherwise noted. The ammonium acetate buffer in which LMWCr is stored had no effect on the assays at concentrations equiva-

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lent to those which result when LMWCr is added to the assays. Ammonium vanadate was used as a phosphatase inhibitor. Membrane fragments (as they contain phosphotyrosine themselves) were removed after the reaction using Microcon 30 microconcentrators (Amicon). Bovine pancreas insulin was from Sigma. Any absorbance contributions to the assay by the addition of LMWCr or metal ions were determined by measuring the background absorbance of the assay in the absence of ATP and membranes or ATP and isolated insulin receptor at each LMWCr or metal ion concentration; these contributions (which were often below detection limits) were substracted from all data. Polyclonal antibodies whose epitope corresponds to amino acids 29−48 mapping at the amino terminus of the precursor form of human insulin receptor α chain were obtained from Santa Cruz Biotechnology. Antibodies (0.7 μg) were incubated with the membranes for 2 h at circa 4 °C prior to the initiation of the reaction by ATP.

Rat liver insulin receptor was obtained from Sigma. The receptor was incubated with insulin for 2 h at circa 4 °C prior to the assay in 67 mM Hepes buffer, pH 7.4, containing 0.06% Triton-X 100, 0.3 M NaCl, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 M sodium acetate, and 100 μg/mL of BSA as described by Petruzzelli et al. (1984). The kinase reaction was allowed to proceed for 75 min at 37 °C after being initiated by addition of ATP to give a final ATP concentration of 0.75 μM.

Phosphotyrosine 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. 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}). Activation of PTP activity by LMWCr was examined as described by Davis et al. (1996).

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), $MnSO_4 \cdot H_2O$ (Mallinckroft), and $Na_2MoO_4 \cdot 2H_2O$ (Fisher). Metals were added to the reaction mixture and incubated for 10 min at 37 °C prior to reaction initiation by addition of ATP.

Miscellaneous. All visible spectroscopic measurements were made with a Hewlett-Packard 8451A diode array spectrophotometer. Fluorescence measurements were obtained with a Perkin Elmer 204 fluorescence spectrophotometer. All kinetics experiments were performed in plasticware and in triplicate and reproduced at least once. Errors are presented throughout including all tables and graphs as standard deviations (1σ) of the triplicate analyses. Similarly, all Cr and LMWCr concentration determinations were made in triplicate. Curves in all figures represent best fit hyperbolic curves. Doubly deionized water was used in all operations.

RESULTS AND DISCUSSION

Kinase Studies

The addition of bovine liver LMWCr to rat adipocytic membranes in the presence of 100 nM insulin results in a concentration-dependent 3.5−8-fold stimulation of insulin-dependent protein tyrosine kinase activity using a fragment of gastrin and cell division kinase $p34^{cdc2}$ as substrates, respectively, while no activation of kinase activity with either substrate is observed in the absence of insulin (Figure 1). The dependence of the activations on the concentration of LMWCr can be fit to a hyperbolic curve to give dissociation constants (K_m's) of approximately 875 pM. Addition of polyclonal antibodies whose epitope corresponds to amino acids 29−48 mapping at the amino terminus of the precursor form of the human insulin receptor α chain (the region contains amino acid residues believed to be essential in the binding of insulin to the α subunit; the α subunits of insulin receptor are external to the cell membrane) (Lee & Pilch, 1994) to the rat adipocytic membranes in the presence of 100 nM insulin results in a loss of the activation of insulin receptor kinase activity (Figure 2). (No visible evidence for precipitation was present after addition of antibody to membranes; addition of antibodies also does not result in a loss of non-insulin-dependent kinase activity, suggesting no significant precipitation or aggregation of membranes was occurring. After treatment with antibodies, membrane fragments were also tested for phosphotyrosine phosphatase activity; no effect as a result of addition of antibodies was observed. Similarly, LMWCr was able to stimulate this PTP activity in an identical fashion in antibody-treated and control

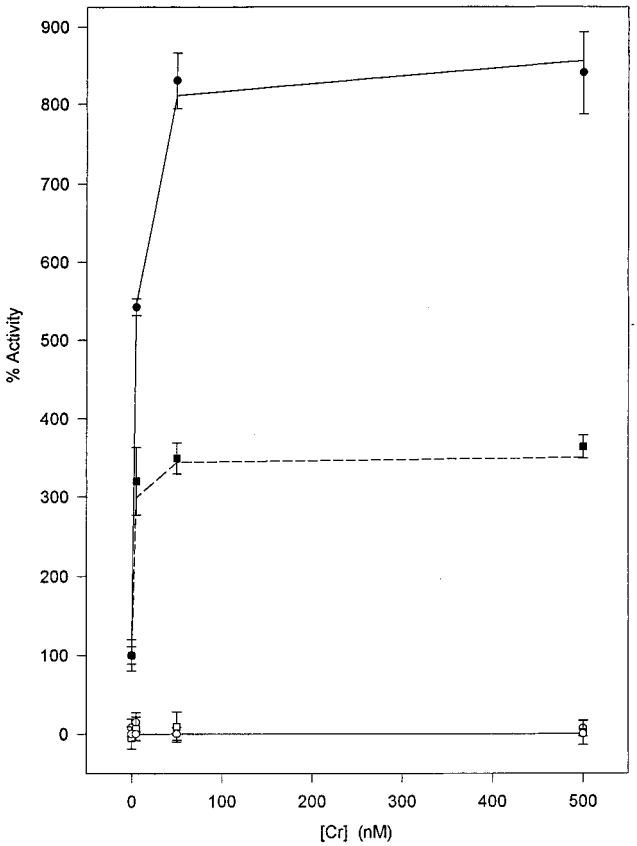

FIGURE 1: Activation of rat adipocytic membrane protein tyrosine kinase activity using 0.75 μM fragments of cell division kinase $p34^{cdc2}$ (amino acids 6−20) (circles) and gastrin (amino acids 1−17) (squares) as substrates by bovine liver LMWCr in the presence (solid symbols) or absence (open symbols) of 100 nM insulin. 0% activity corresponds to kinase activity in the absence of insulin; this value of the total membrane kinase activity is typically about 0.208 pmol of phosphotyrosine per mg of membranes. 100% activity corresponds to insulin-stimulated kinase activity in the absence of LMWCr and is typically about 0.338 pmol of phosphotyrosine per mg of membranes. 25 μL of a rat adipocyte membrane suspension corresponding to 310 μg of protein/μL was utilized.

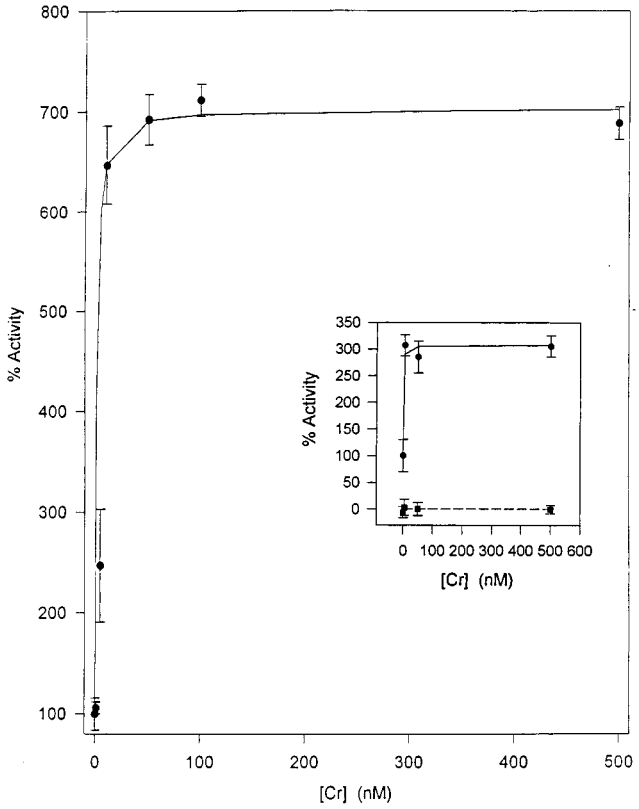

FIGURE 2: Activation of protein tyrosine kinase activity of isolated rat liver insulin receptor by bovine liver LMWCr using a fragment of gastrin (0.75 μM) as substrate in the presence of 100 nM insulin. The inset shows the activation of protein tyrosine kinase activity of rat adipocytic membranes in the presence (squares) and absence (circles) of polyclonal antibodies whose epitope corresponds to amino acids 29−48 mapping at the amino terminus of the precursor form of human insulin receptor α chain using a fragment of gastrin (0.75 μM) as substrate in the presence of 100 nM insulin by LMWCr. 25 μL of a rat adipocytic membrane suspension corresponding to 40 μg of protein/mL was utilized. The curve was fit using a dissociation constant of 875 pM.

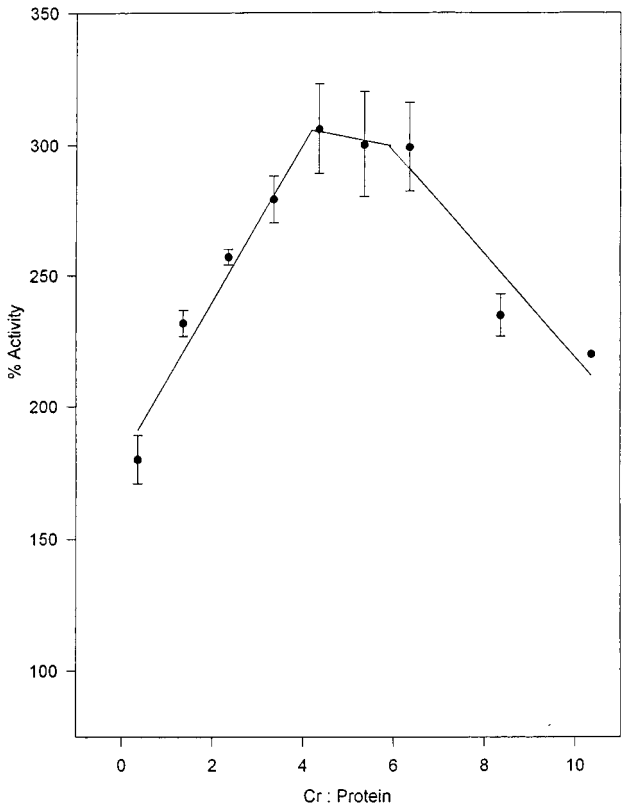

FIGURE 3: Chromic ion titration of the ability of bovine liver apoLMWCr (125 nM) to activate protein tyrosine kinase activity in rat adipocytic membranes in the presence of 100 nM insulin using 0.75 μM gastrin fragment as substrate. 25 μL of a rat membrane suspension corresponding to 40 μg of protein/mL was utilized.

membrane fragments. Incubation of membrane fragments with 0.7 μg of BSA instead of the polyclonal antibodies had no effect on the tyrosine kinase assays versus controls.) Thus, LMWCr significantly potentiates a membrane kinase activity only in the presence of insulin, and preventing the binding of insulin to its receptor eliminates the ability of LMWCr to potentiate kinase activity. Given that the membrane preparations should be devoid of soluble, physiological insulin receptor kinase substrates, such as insulin receptor substrate 1 (IRS-1) (White & Kahn, 1994), these results suggest that the site of action of LMWCr is the insulin receptor itself.

This hypothesis was tested by examining the activation of isolated rat insulin receptor by bovine liver LMWCr in the presence of insulin. As shown in Figure 2 using gastrin fragment as substrate, the addition of bovine liver LMWCr to isolated and purified rat liver insulin receptor amplifies the stimulation of receptor protein tyrosine kinase activity by insulin approximately 7-fold. Fitting the activation curve to a hyperbolic function indicates that LMWCr interacts with the insulin-activated receptor with a dissociation constant of approximately 250 pM. Insulin receptor is the site of LMWCr's action.

Chromium plays a crucial role in the activation of insulin receptor kinase activity by LMWCr. ApoLMWCr (i.e., metal-free) displays little ability to activate insulin-dependent tyrosine kinase activity in the rat adipocyte membranes (Figure 3). [Apoprotein samples used in this study contained circa 0.3 Cr; while most of this remaining Cr is in the form of EPR detectable mononuclear centers (Davis et al., 1996), a tiny fraction of the protein is in the form containing four chromic ions giving rise to this residual activity. A similar result has observed in PTP activation studies (Davis et al., 1996).] However, titration of apoLMWCr with chromic ions results in the total restoration of the ability to activate kinase activity; approximately four chromic ions per oligopeptide are required for maximal activity. This is consistent with the number of chromium (four per oligopeptide) reported to be bound to holoLMWCr from liver sources (Yamamoto et al., 1987, 1988; Davis & Vincent, 1997). Similarly, maximal activation of a rat adipocyte phosphotyrosine protein phosphatase by LMWCr requires four chromium per oligopeptide (Davis et al., 1996), while the ability of LMWCr to stimulate the insulin-dependent conversion of glucose to carbon dioxide by rat adipocytes has also been reported to be directly dependent on the chromium content of LMWCr (Yamamoto et al., 1989). Addition of more than six chromium per oligopeptide results in inhibition of kinase activation. This reconstitution of LMWCr's activation potential is specific to chromium. Transition metal ions other than chromium which are commonly associated with biological systems are ineffective in potentiating the ability of apoLMWCr to activate kinase activity (Figure 4) in the presence of insulin; in fact, all the ions except Cr^{3+} resulted in loss of activation potential relative to apoLMWCr. Similarly, the metal ions themselves (in the absence of apoLMWCr) are ineffective in activating the insulin-dependent kinase activity. Thus, the ability of LMWCr to potentiate the effects of insulin in

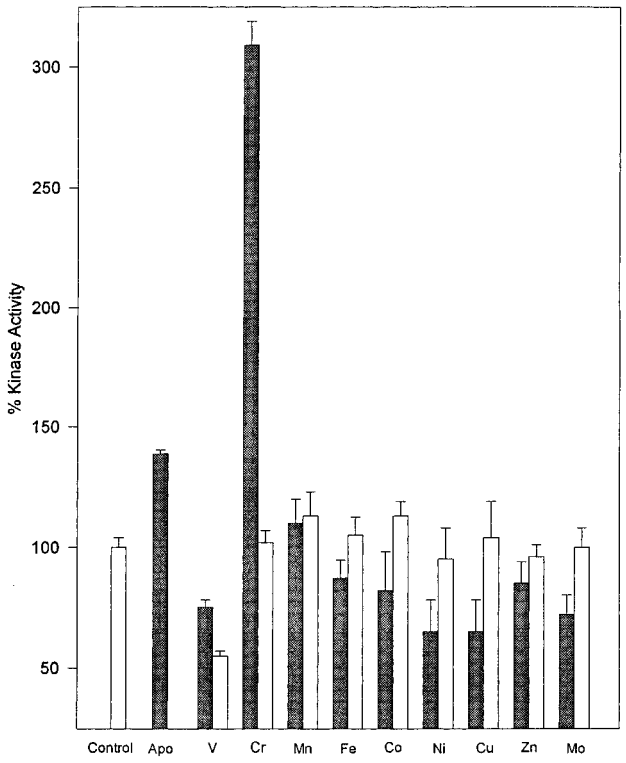

FIGURE 4: Activation of rat adipocytic membrane protein tyrosine kinase activity using 0.75 μM gastrin fragment as substrate by metal ions (500 nM) (open bars) or by apoLMWCr (125 nM) and metal ions (500 nM) (shaded bars) in the presence of 100 nM insulin. 25 μL of a rat adipocytic membrane suspension corresponding to 40 μg of protein/mL was utilized. The control contains no added apoLMWCr or metal ions.

stimulating the insulin-dependent protein tyrosine kinase activity of insulin receptor is specific to chromium and is directly dependent on the chromium content of LMWCr.

Conclusions

The results of this and other related studies are beginning to elucidate the biological function of chromium and LMWCr, which bears striking similarities to the role calmodulin. [Calmodulin binds four calcium ions in response to a calcium flux; the tetracalcium form then binds to kinases and phosphatases stimulating their activity (Babu et al., 1988).] Recent blood homeostasis studies have revealed that in response to increases in blood insulin concentrations chromium concentrations decrease as the metal is taken up by insulin-dependent cells (Morris et al., 1992, 1993a,b). LMWCr is maintained in these cells almost entirely in its apo (metal-free) form (Yamamoto et al., 1987). However, apoLMWCr has a large chromic ion binding constant; for example, LMWCr can remove Cr from Cr−transferrin (Yamamoto et al., 1984, 1987). The ability of LMWCr to potentiate the effects of insulin or activate phosphotyrosine phosphatase or protein tyrosine kinase activity is directly dependent on its Cr(III) content (Davis et al., 1996; Yamamoto et al., 1989) and cannot be replaced by other transition metal ions (Davis et al., 1996). Thus, movement of chromium from the blood to insulin-sensitive tissues results in the formation of the holoLMWCr, possessing four chromic ions in a tetranuclear assembly (Vincent, 1994b; Davis et al., 1996). HoloLMWCr then binds with a dissociation constant on the order of 100 pM to 10 nM to at least protein tyrosine kinases and phosphotyrosine phosphatases, whose activities are subsequently stimulated. Thus, insulin appears to stimulate an activation of LMWCr by initiating its loading with Cr, which in turn can potentiate insulin's effects. Therefore, it is not surprising that Cr deficiency has been found to be associated with non-insulin-dependent diabetes and its symptoms (Anderson, 1986, 1992; Morris et al., 1988; Morgan, 1972) and that, for example, Cr administration to streptotozotocin-induced diabetic rats resulted in enhanced insulin responsiveness while the insulin receptor number remained constant (Yoshomoto et al., 1992). What is surprising is that for the first-row transition elements from vanadium to zinc, which are each essential for some form of life, chromium is the only element for which at least one metallobiomolecule containing the element had not been well characterized in terms of its function and mode of action (Kaim & Schwederski, 1994). In summary, the symptoms of adult-onset diabetes associated with chromium deficiency and attributed to apparent insulin resistance may stem from insufficient quantities of chromium to generate holoLMWCr in response to insulin, such that insulin receptor kinase activity is not activated to an appropriate degree.

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