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COVALENT CROSS-LINKING OF INSULIN-LIKE GROWTH FACTOR-1 TO A SPECIFIC INHIBITOR FROM HUMAN SERUM

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Previous studies have shown that a specific inhibitor of insulin-like growth factor (IGF) action in vitro can be isolated from normal human serum and subsequently partially purified on an IGF-affinity column. The ability of the inhibitor to bind the IGFs has now been confirmed directly using covalent cross-linking techniques. When ^{125}I-IGF-1 was cross-linked to inhibitor using disuccinimidyl suberate, five specifically labelled bands were seen on SDS-PAGE and autoradiography. Two bands (MW 21.5 K and 25.5 K) were intensely labelled, whilst the remaining three (MW 37 K, 34K and 18 K) appeared as minor bands only. Inhibitor bioactivity, following further analysis by hydrophobic interaction chromatography or Con A-Sepharose affinity chromatography, was always associated with the presence of the 21.5 K and/or 25.5 K bands. These data describe, for the first time, the structural nature of the IGF inhibitor protein and raise important questions regarding the relationship of the inhibitor to the primary IGF-binding subunit of the native high MW IGF carrier protein of serum.

We have previously reported the isolation and characterisation from human serum of a specific inhibitor of the in vitro actions of the insulin-like growth factor (IGF) family [1]. The inhibitor was shown to be a hydrophobic, acid-stable protein with a molecular weight (MW) of 16000-18000 (16-18 K) as determined by size exclusion chromatography. It specifically inhibited a variety of the in vitro biological actions of the IGFs. Its mechanism of action was presumed to be via direct binding to the IGFs themselves since inhibitor binding to IGF could be demonstrated by the partial purification of the inhibitor on an IGF-Sepharose affinity column [2].

The binding interaction between inhibitor and IGF has now been investigated further using covalent cross-linking techniques. This approach has provided some important data regarding the structural nature of the inhibitor.

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MATERIALS AND METHODS

Partially purified IGF inhibitor was prepared from Cohn fraction IV-I of human serum as reported previously [1]. The inhibitor was further purified by hydrophobic interaction chromatography (HIC) using Octyl-Sepharose (Pharmacia, Uppsala, Sweden). 15 mg inhibitor was applied to a 1.0 x 15 cm (bed volume = 8 ml) Octyl-Sepharose column equilibrated with 1% formic acid. The column was eluted initially with 1% formic acid, and subsequently with a stepwise acetonitrile gradient of 10, 20 and 80%, in 1% formic acid. Aliquots (150-200 µl) of the collected fractions (1.2 ml) were assayed for inhibitory activity, and the fraction with peak activity was used for covalent cross-linking.

Assay for inhibitor activity

Inhibitor activity was assessed as described in detail previously [1,2] by its ability to inhibit IGF-stimulated [^{14}C]-glucose incorporation into [^{14}C]-lipid by isolated rat epididymal adipocytes [3].

Preparation of ^{125}I-IGF-1

Purified IGF-1, a gift from Dr E.M. Spencer, San Francisco, U.S.A., was iodinated by the Iodogen method [4] and the reaction mixture purified on a Sephadex G-50 column (1.0 x 20 cm) equilibrated in 50 mM Tris/HCl pH 7.5. The peak tube of the iodinated protein fraction was then repurified prior to cross-linking studies on an Octyl-Sepharose column as described by Baxter and Brown [5].

Covalent cross-linking

100 µg of partially purified inhibitor in a final volume of 250 µl 0.1 M sodium phosphate, pH 7.4 was incubated for 4 h at room temperature (20°C) with 300,000 cpm of ^{125}I-IGF-1 (+ excess unlabelled IGF for determination of specific binding). Disuccinimidyl suberate (Pierce Chemical Co., IL, U.S.A.), in a minimal volume of dimethylsulphoxide (Sigma, St Louis, MO, U.S.A.), was then added to a final concentration of 1 mM, and the cross-linking reaction allowed to proceed for 30 min at room temperature. The reaction was terminated by the addition of electrophoresis sample buffer (described below) and the cross-linked samples analysed by SDS-PAGE and autoradiography.

Electrophoresis and Autoradiography

Samples were boiled for 3 min in the presence of 65 mM Tris-HCl, pH 6.8, 3% sodium dodecylsulphate, 10% glycerol with or without 100 mM dithiothreitol (BioRad, Sydney, Australia), and then subjected to electrophoresis on 15% polyacrylamide gels (30: 0.8 w/w acrylamide: bisacrylamide) according to the protocol of Laemmli [6]. Gels were fixed and stained in 50% trichloroacetic acid containing 0.1% Coomassie blue R-250, dried, and autoradiographed by exposure to Kodak XAR-5 film using Dupont Lightning Plus enhancing screens at -70°C for 3-7 days. Molecular weight marker proteins were obtained from Pharmacia (Uppsala, Sweden), and the MW of specifically-labelled bands was determined by graphic interpolation.

Concanavalin A (Con A)-Sepharose Affinity Chromatography

8 mg inhibitor was applied to a 1.5 x 15 cm (bed volume = 15 ml) Con A-Sepharose column (Pharmacia, Uppsala, Sweden), and then washed with 0.02 M Tris/HCl, pH 7.4 containing 0.5 M NaCl, 1mM $MnCl_2$, and 1 mM $CaCl_2$. Bound substances were eluted subsequently with 0.4 M α-D-manno pyranoside (Calbiochem-Behring, Sydney, Australia) in the starting buffer. Unbound and bound fractions were pooled separately, dialysed in Spectrapor 3 tubing (Spectrum Medical Industries, California, U.S.A.) against water overnight and lyophilised.

RESULTS AND DISCUSSION

When partially purified inhibitor was cross-linked to ^{125}I-IGF-1, five cross-linked complexes were seen on autoradiography (Fig.1). These complexes were specific for IGF since addition of 0.25 mU insulin equivalents of unlabelled IGF to the incubation (Fig.1, lanes B and D) significantly reduced the intensity of labelling of the observed bands. The two most intensely labelled bands had MW of 25.5 K and 21.5 K, whilst the three minor bands had MW of 37 K, 34 K and 18 K. Reduction of the cross-linked samples with 100 mM dithiothreitol (Fig. 1, lanes C and D) did not significantly affect the electrophoretic migration of any of the cross-linked complexes, indicating the absence of any major disulphide linkages in these complexes.

As indicated in Fig.2, further purification of the inhibitor was achieved by HIC, using a stepwise 0-80% acetonitrile gradient on

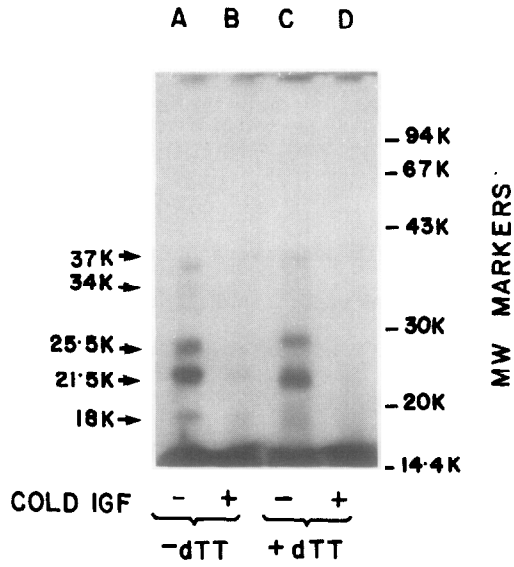
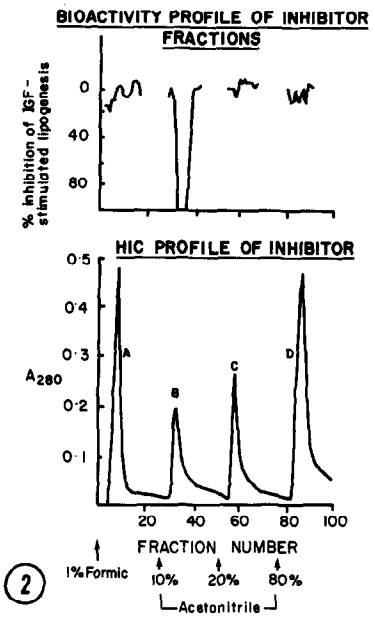


Fig. 1. Autoradiograph of ^{125}I-IGF-I covalently cross-linked to a partially purified inhibitor preparation [1]. Inhibitor was incubated with ^{125}I-IGF-I in the absence (lanes A,C) and presence (lanes B,D) of unlabelled IGF (0.25 mU). Following cross-linking with disuccinimidyl suberate the covalent complexes were analysed by SDS-PAGE and autoradiography under non-reduced (lanes A,B) or reduced [100 mM dithiothreitol (dTT), lanes C,D] conditions. MWs of specifically labelled bands are indicated by the arrows. The same pattern was observed in 7 experiments.



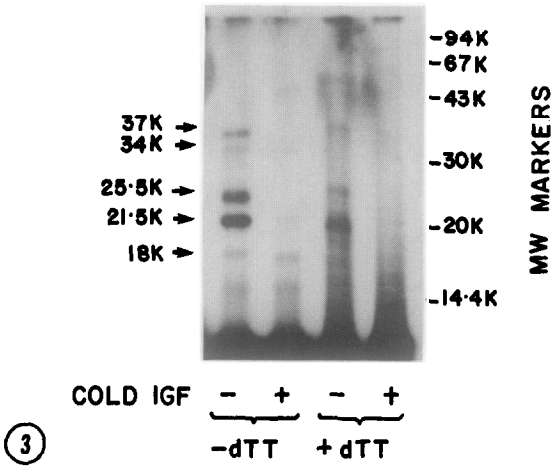


Fig. 2. Hydrophobic interaction chromatography of partially purified inhibitor on Octyl-Sepharose. Inhibitor was applied to an Octyl-Sepharose column and eluted with a stepwise acetonitrile gradient as described under Methods. The protein ($A_{280\ nm}$) profile is shown in the lower panel; the inhibitor bioactivity profile of eluted fractions is shown in the upper panel. Inhibitor bioactivity is expressed as the percent inhibition of IGF-stimulated lipogenesis as described under Methods. The same data were obtained in more than 10 experiments.

Fig. 3. Autoradiographs of ^{125}I-IGF-I covalently cross-linked to Octyl-Sepharose purified inhibitor (peak tube of fraction B of Fig. 2). The data, presented as described for Fig. 1, were identical in two such experiments.

Octyl-Sepharose. Five protein peaks were obtained, with one peak corresponding to each elution step. Inhibitor bioactivity was present only in the peak eluted by 10% acetonitrile in 1% formic acid (peak B). Cross-linking of the peak tube of the bioactive fraction gave a similar pattern (5 specific bands) to that obtained for the partially purified inhibitor (Fig.3). The 18 K band however did not appear to be specific in this case, since addition of excess unlabelled IGF did not abolish the labelling. This inconsistency in the specificity of the labelling of the 18 K band was observed in other cross-linking experiments.

As one approach to further elucidating the nature of the specifically cross-linked complexes, partially purified inhibitor was

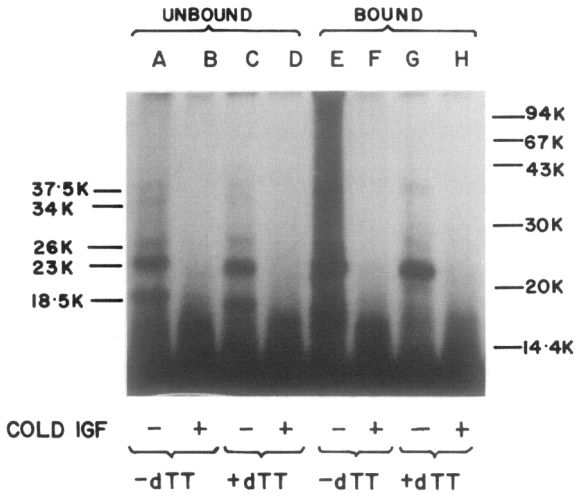


Fig. 4. Autoradiograph of ^{125}I-IGF-I cross-linked to partially purified inhibitor fractionated by Con A-Sepharose affinity chromatography. The Con A-bound fraction was eluted with α-D-mannopyranoside. Both bound and unbound fractions possessed inhibitor bioactivity (not shown). The data are presented as described for Fig. 1. Three such experiments were performed.

subjected to Con A-Sepharose affinity chromatography prior to cross-linking. Inhibitor bioactivity was detected in both the bound and unbound fractions (data not shown). On cross-linking, the unbound (non Con A-reactive) fraction gave five specifically labelled bands (MW 37.5 K, 34 K, 26 K, 23 K, 18.5 K) (Fig. 4), similar to those obtained for the original inhibitor preparation. The most intensely labelled complexes, however, were the 23 K and 18.5 K bands. The bound fraction (Con A-reactive) gave a major, intensely labelled complex at 23 K, with faintly labelled complexes at 26 K and 37.5 K. The presence of the 37.5 K, 26 K and 23 K complexes in both the bound and unbound fractions was unexpected but was not due to problems associated with column overloading, since re-chromatography of the unbound fraction gave similar results. An explanation may be slight differences in the glycosylation state of the various IGF-binding proteins. An important observation, however, in both cross-linking studies reported here is the association of IGF inhibitor activity

with the presence of one or both of the two intensely labelled bands of MW 21.5 K and 25.5 K.

Assuming a 1:1 binding stoichiometry with IGF (MW 7.5 K), the two major, intensely labelled complexes observed for the inhibitor (MW 25.5 K and 21.5 K) represent binding proteins of MW 18 K and 14 K respectively, very close to the MW of the inhibitor (18-16 K) determined previously by gel filtration [1]; the two minor complexes of MW 37 K and 34 K represent binding proteins very similar in size to some of the classical IGF-carrier proteins such as those isolated from human amniotic fluid [7]. The significance of the 18 K complex is as yet unclear.

Recently, using similar cross-linking techniques, Wilkins and D'Ercole [8] reported the presence of a number of specifically cross-linked IGF-complexes in whole human plasma, including complexes of MW 35-43 K and 24-28 K. Similar observations have been made in other recent studies using normal human serum [9,10] and human cord plasma [11]. A major conclusion drawn by Wilkins and D'Ercole [8] was that the 24-28 K complexes were derived directly from higher MW carrier proteins, and this led them to speculate that the 24-28 K complexes were the primary subunits from which the high MW (~150 K) native oligomeric carrier protein was formed. The similarity in size of this carrier protein subunit with the major bands (21.5 and 25.5 K), observed in our inhibitor-IGF cross-linking studies is striking, and raises the possibility that the IGF inhibitor may represent the primary IGF-binding subunit of the native MW ~150 K IGF-carrier protein of serum. This possibility is supported by recent observations (G.T. Ooi and A.C. Herington, unpublished data) that in vitro IGF inhibitory activity can be generated by prolonged acid treatment from an otherwise non-inhibitory high MW, classical IGF-carrier protein fraction. These data together, raise intriguing issues regarding the possible physiological role for the inhibitory IGF-binding proteins when present in serum either as a monomeric

subunit (MW 14-18 K) or as part of the native carrier protein complex (MW ∿150 K).

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