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Identification and molecular characterization of a high-affinity cardiomyocyte transforming growth factor-β2 receptor

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Rat neonatal heart muscle cells (cardiomyocytes) were found to express a high-affinity surface receptor for transforming growth factor-β2 (TGF-β2). Specific binding was rapid, saturable, ligand-selective, and reversible. Equilibrium binding analyses revealed that the cardiomyocyte had one class of specific binding sites with a $K_d \leq 26$ pM TGF-β2, a B_{max} of ~9 fmol/10^6 cells, and ~5,000 binding sites/cardiomyocyte. Binding was selective for TGF-β2 in comparison to other TGF-β isoforms and to unrelated growth factors. Affinity-binding experiments revealed three types of cardiomyocyte TGF-β2 binding proteins, the most prominent of which corresponded to the high-molecular mass proteoglycan. These data raise the possibility that the anti-ischemic cardioprotective effects of TGF-β may reflect receptor-mediated signal transduction at the cardiomyocyte level.

Transforming growth factor-β; Cardiomyocyte; Heart muscle; Cytokine; Receptor; Affinity labeling

1. INTRODUCTION

The peptide cytokine transforming growth factor-β (TGF-β) exists as a family of three highly conserved mammalian isoforms (TGF-β1, -β2, and -β3) with significant (~70%) sequence homology and structural similarity [1]. Although initially described in transformed cells, TGF-β is synthesized in many tissues. The multiple effects of TGF-β on target cells are mediated by specific, high-affinity TGF-β receptors, the binding to which is the first step in a poorly understood signal-transduction cascade [2]. The distribution and characteristics of cellular TGF-β receptors are, consequently, topics of great current interest [2].

The cardiovascular system is both a source of TGF-β and a site of action of this cytokine. TGF-β has been detected in the vessel wall, circulating blood cells, and heart muscle [3]. Cardiomyocytes secrete TGF-β which can associate with the cellular membrane [4]. One of the most important cardiovascular aspects of TGF-β is its salutary effects against myocardial ischemia [5]. Although the anti-ischemic cardioprotection has been hypothesized to reflect support of heart muscle contraction [4], endothelial preservation [5], and/or an antioxidant effect [6], detailed knowledge of TGF-β's physiology in normal, let alone ischemic, heart muscle is lacking. A direct relationship between cardiomyocyte injury and TGF-β is implied by the changes in myocardial TGF-β content/gene expression during ischemia and post-ischemic reperfusion [3].

As detailed in a recent review [7], little direct information is available regarding cardiomyocytes as peptide growth factor targets. We report the initial identification and characterization of a specific receptor population responsible for TGF-β2 binding to neonatal rat cardiomyocytes. Our results constitute the first biochemical analysis of cardiomyocyte TGF-β receptors and provide a context for potential TGF-β2 signal transduction in heart muscle.

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Abbreviations: aFGF, acidic fibroblast growth factor; BME, Eagle's basal medium; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; EDTA, ethylene diaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PDGF, platelet derived growth factor; PMSF, phenylmethanesulfonyl fluoride; TGF-β, transforming growth factor-β; Tris, tris(hydroxymethyl)aminomethane.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine serum albumin, essentially fatty acid-free (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), ethylene diaminetetraacetic acid (EDTA), protease inhibitors, and miscellaneous biochemicals were obtained at the highest available grade from Sigma (St. Louis, MO). Protease inhibitor cocktail I was an aqueous solution of 1 mg/ml leupeptin, 1 mg/ml antipain, 5 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, and 10 mg/ml benzamidine hydrochloride. Protease inhibitor cocktail II consisted of 1 mg/ml pepstatin, 1 mg/ml bestatin, and 30 mM phenylmethanesulfonyl fluoride (PMSF) in dimethylsulfoxide. Disuccinimidyl suberate (DSS) was from Pierce Chemical Co. (Rockford, IL). [^{125}I]TGF-β2 (1,800–2,200 Ci/mmol, spec. act.) was a human recombinant product [8] iodinated as detailed [9]. Human recombinant TGF-β3 was provided by N. Cerletti (CIBA-GEIGY, Basel, Switzerland). TGF-β1 and platelet-derived growth factor (PDGF) were from human platelets, and acidic fibroblast

growth factor (aFGF) was from bovine brain (R & D Systems, Minneapolis, MN)

2.2 *Cardiomyocyte culture*

Hearts were obtained from 3-day-old Sprague–Dawley rats, and cardiomyocytes were isolated by external digestion of left-ventricular myocardium [10,11]. The isolated cardiomyocytes were seeded in complete Eagle's basal medium (BME) (5×10^5 cells/35-mm well) and incubated at 37°C under 95% air/5% CO_2 [10]. Confluency was reached within 72 h post-plating, at which time the cells were used experimentally as a monolayer network of synchronously beating cardiomyocytes containing 109 ± 5 μg cell protein/well (mean ± S.D.: $n = 10$). The experimental cultures consisted almost exclusively (>95%) of ventricular cardiomyocytes [11].

2.3 *Equilibrium binding studies*

Cardiomyocyte monolayers were preincubated overnight (i.e. for 18 h) in serum-free medium; the duration of the serum-free preincubation (from 2 to 24 h) did not alter the subsequent [^{125}I]TGF-β2 binding (data not shown). The monolayers were then washed with 6 ml ice-cold binding buffer (serum-free BME; 0.1% (w/v) BSA, 25 mM HEPES, pH 7.4) Each washed monolayer was incubated with [^{125}I]TGF-β2 in 1.0 ml ice-cold binding buffer at 4°C with continuous gentle agitation. The concentrations of [^{125}I]TGF-β2 used and the incubation times were as indicated in the text and figure legends. The binding assay was terminated by removing the [^{125}I]TGF-β2-containing binding buffer and washing each labeled monolayer with 6 ml ice-cold binding buffer. The cells were then solubilized with 0.6 ml solubilization buffer (20 mM HEPES, pH 7.4, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0 01% (w/v) BSA), and triplicate aliquots were counted for ^{125}I label. Non-specific binding was taken as the [^{125}I]TGF-β2 which became cell-associated during the binding assay in the presence of a 1,000-fold molar excess of unlabeled ligand. For competition studies, the binding assay was conducted with 40 pM [^{125}I]TGF-β2 in the presence of varying concentrations of unlabeled TGF-β1, -β2, -β3, PDGF, or aFGF. For displacement studies, equilibrium binding was established with 4, 20, or 40 pM [^{125}I]TGF-β2, and the labeled cardiomyocytes were then incubated with a 1,000-fold molar excess of unlabeled TGF-β2 for various periods of time under binding assay conditions.

2.4. *TGF-β2 internalization assay*

Cardiomyocytes were incubated with 40 pM [^{125}I]TGF-β2 for 30 min and processed as for equilibrium binding (above). The washed cells were then lysed with hypotonic buffer (5 mM HEPES, 1 mM $MgSO_4$, pH 7.4), and cytoplasmic and membrane fractions were prepared [11]. The subcellular fractions were counted for associated ^{125}I label.

2.5. *Affinity labeling of cardiomyocyte TGF-β2 binding proteins*

Confluent cardiomyocyte monolayers were incubated with [^{125}I]TGF-β2 under equilibrium-binding conditions (above) to generate a cell monolayer with ligand-occupied TGF-β2 receptors. After incubation with [^{125}I]TGF-β2, each monolayer was washed with 6.0 ml binding buffer and then rinsed with 1.0 ml ice-cold binding buffer lacking BSA. The labeled cells were incubated at 4°C for 15 min with BSA-free binding buffer containing 135 μM DSS. Each monolayer was then rapidly rinsed with 1.0 ml ice-cold detachment buffer (10 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.3 mM PMSF) and harvested into 1 ml of this buffer supplemented with 10 μl/ml of each protease inhibitor cocktail. Samples were solubilized in buffer (10 mM Tris, pH 7.0, 125 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10 μl/ml of each protease inhibitor cocktail) and prepared for SDS-PAGE according to Laemmli [12] on 7% (w/v) polyacrylamide slab gels, which were processed for autoradiography as detailed [13].

2.6. *Data evaluation*

The ReceptorFit program (London Software, Chagrin Falls, OH) [14] was used for progressive-complexity analysis of the binding data and for Klotz transformations [15]. The program features weighted non-linear least-squares regression Statistical significance of the data-fit to a given receptor model was in accordance with F-test criteria at $P \leq 0.01$. Scatchard [16] and isotherm (i.e. bound vs. free) plots were generated with Cricket Software (Malvern, PA).

3. RESULTS

We initially assessed the interaction of TGF-β2 with the cardiomyocyte for characteristics indicative of a specific TGF-β2 receptor by incubating the cells under serum-free conditions with selected concentrations of [^{125}I]TGF-β2 for 30 min or 40 pM [^{125}I]TGF-β2 for discrete intervals up to 60 min. These experiments revealed a concentration-dependent association of [^{125}I]TGF-β2 with the cardiomyocyte (Fig. 1). The difference between cardiomyocyte-associated [^{125}I]TGF-β2 in the absence and presence of a 1,000-fold molar excess of unlabeled TGF-β2 defined a saturable, specific-binding component, which plateaued in a time-dependent manner (Fig. 1, inset). The plateau did not reflect a ligand deficit, for the specifically bound [^{125}I]TGF-β2 at 30 min was some 18-fold less than that available from the 40 pM [^{125}I]TGF-β2 supplied, and total (i.e. non-specific) binding continued to increase after specific binding had become saturated. Consequently, prolonged (up to 3 h) binding incubations did not increase

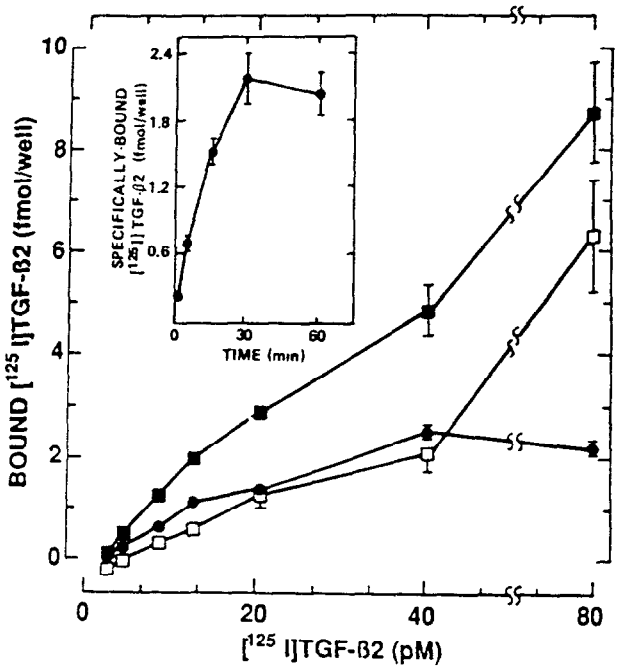

Fig. 1. Concentration- and time-dependence of cardiomyocyte TGF-β2 binding. Confluent cardiomyocyte monolayers (5×10^5 cells/well) were incubated for 30 min at 4°C in binding buffer containing 2–80 pM [^{125}I]TGF-β2 or for up to 60 min with 40 pM [^{125}I]TGF-β2 (inset). Parallel incubations were carried out in the presence of a 1,000-fold molar excess of unlabeled TGF-β2 to assess non-specific binding. At the end of each incubation, the cardiomyocytes were recovered, and the cell-associated [^{125}I]TGF-β2 was quantified. Specific binding (●) represents the calculated difference between total (■) and non-specific (□) binding. Data are means ± S.E.M. ($n \geq 6$ independent cultures).

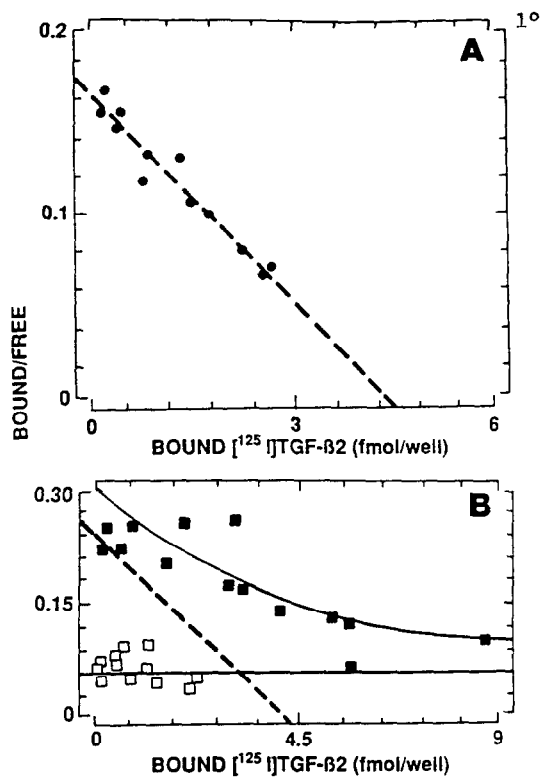

Fig. 2. Scatchard (A) and iterative non-linear least-squares regression (B) analyses of concentration-dependent cardiomyocyte TGF-β2 binding data from experiments of the type depicted in Fig. 1. The dashed lines (---) represent regression (A) and non-linear least squares (B) fits of the binding data. For B, the total (■) and measured non-specific (□) binding data incorporated into the model are displayed. The data are derived from three independent cultures, each value the average over three wells. The range about each mean was <10%.

specific binding, but resulted in total binding which was largely non-specific (data not shown). In accord with the propensity of TGF-β to adhere to surfaces [17], non-specific binding averaged some 35% of total after a 30 min binding assay with 40 pM [^{125}I]TGF-β2, a proportion observed in other cell types [18]. Under the binding conditions employed, cardiomyocyte-associated [^{125}I]TGF-β2 was completely recovered in the particulate membrane fraction prepared from a hypotonic cell lysate; ligand internalization appeared insignificant, for negligible ^{125}I label was detected in the soluble cytoplasmic fraction.

Scatchard analysis [16] of concentration-dependent binding data from experiments of the type depicted in Fig. 1 indicated that the neonatal rat cardiomyocyte displayed one class of specific TGF-β2 binding sites with a K_d of ~26 pM, a B_{max} of 8.8 fmol/10^6 cardiomyocytes, and, maximally, 5,290 binding sites/cell (Fig. 2A). Inherent limitations associated with applying Scatchard formulations to equilibrium-binding data may generate gross quantitative errors in apparent receptor number/affinity [15]. We therefore evaluated the cardiomyocyte TGF-β2 equilibrium-binding data in a more rigorous manner by testing their fit to progressively more complex models using iterative non-linear least-squares regression [14]. Although the cardiomyocyte [^{125}I]TGF-β2 binding data were incompatible with any multi-site model tested, they did fit a one-site model regardless of whether measured (Fig. 2B) or calculated (data not shown) non-specific binding had been used to derive specific binding. As summarized in Table I, Scatchard analysis gave somewhat higher mean K_d, B_{max}, and receptor-density values than did the iterative analysis. Better fit with less variability was achieved when the measured non-specific binding was used in the iterative analysis (Table I), likely reflecting a greater inherent error in the computed theoretical non-specific binding for a ligand such as TGF-β having a tendency to adhere to surfaces [17].

A Klotz transformation [15] of the concentration-dependent binding data in Fig. 2 yielded a K_d of ~22 pM TGF-β2 (Fig. 3), in good agreement with the K_d values from the Scatchard and non-linear least-squares regression analyses (cf. Table I). The distribution of binding values on either side of the Klotz-plot inflection point demonstrated that the [^{125}I]TGF-β2 concentration range studied adequately bracketed the receptor K_d [15].

Picomolar concentrations of unlabeled TGF-β2 effec-

Table I

Characterization of the cardiomyocyte TGF-β2 receptor population

Analysis	Receptor parameter			
	Best-fit model	K_d (pM)	B_{max} (fmol/10^6 cells)	Receptor density (number/cardiomyocyte)
Scatchard	One-site	26	8.8	5,290
Non-linear least-squares regression				
Measured non-specific binding	One-site	17.3 ± 3 4	8.5 ± 0.6	5,120 ± 480
Computed non-specific binding	One-site	12.2 ± 5.3	6.7 ± 1.9	4,030 ± 1,023

Characteristics of the cardiomyocyte TGF-β2 receptor population were derived as detailed in Fig. 2 from concentration-dependent binding data obtained in experiments of the type depicted in Fig. 1. Tabulated data are mean values for a typical experiment over ≥3 independent cultures (Scatchard analysis) or mean values ± S.E.M. (n = 6 independent cultures) (least-squares regression analysis).

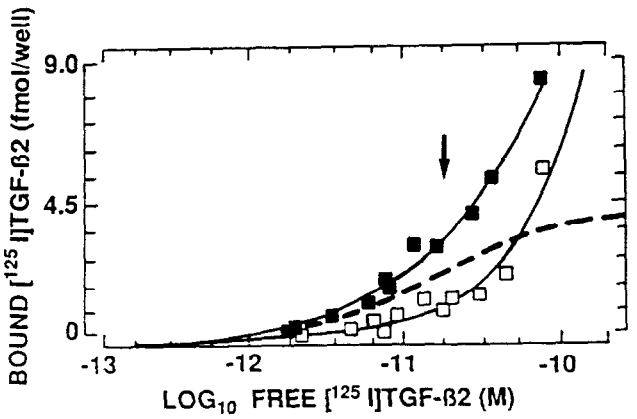

Fig 3. Klotz analysis of cardiomyocyte TGF-β2 binding The total (■) and measured non-specific (□) binding data (Fig. 2) were used to generate a semi-logarithmic Klotz transform (---). The arrow marks the calculated inflection point of the Klotz plot, yielding an estimated K_d value of 22 pM

tively competed for [^{125}I]TGF-β2 specific binding in standard incubations with 40 pM [^{125}I]TGF-β2 and 5×10^5 cardiomyocytes; 50% inhibition of [^{125}I]TGF-β2 binding (IC_{50} value) required 250 pM TGF-β2 (Fig. 4). In comparison, TGF-β1 weakly competed for [^{125}I]TGF-β2 binding with an IC_{50} of ~2 nM. TGF-β3 was only slightly competitive (IC_{50} > 10 nM). High (4 nM) concentrations of unrelated ligands (PDGF, aFGF) were not competitive. Specifically bound [^{125}I]TGF-β2 could be rapidly and completely displaced by excess unlabeled ligand (Table II).

In order to visualize the cardiomyocyte TGF-β2 binding protein(s), [^{125}I]TGF-β2 was cross-linked to cell monolayers, and the protein labeling was examined by

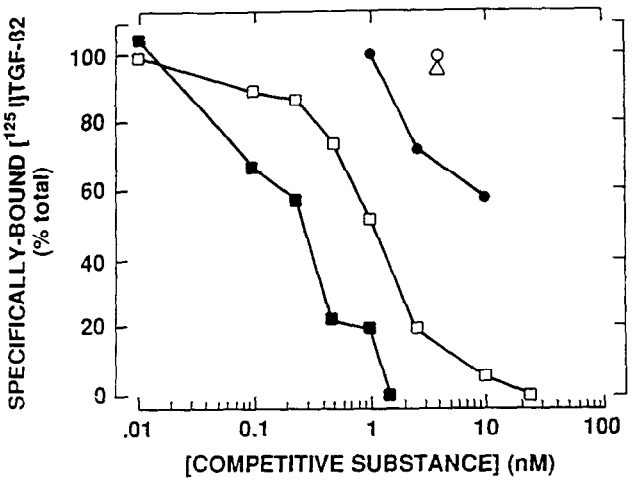

Fig. 4. Competition of cardiomyocyte [^{125}I]TGF-β2 specific binding by unlabeled TGF-β1 (□), TGF-β2 (■), TGF-β3 (●), PDGF (○), or aFGF (△). Specific binding is expressed relative to that in contemporaneous cultures not containing unlabeled test substance. The data are from a representative experiment on 3 independent cultures; the range about each mean was <8%.

SDS-PAGE followed by autoradiography. The cross-linking pattern obtained was similar to that reported for other cell types [19] in that it showed three major TGF-β2 binding proteins (Fig. 5). Most of the [^{125}I]TGF-β2 was associated with a ~280-kDa complex, probably the type III cell surface proteoglycan [19]. Two other binding proteins with apparent molecular masses of ~55 kDa and ~85 kDa probably correspond to the type I and type II receptors, respectively [1,19]. SDS-PAGE analysis of cell membranes isolated from affinity-labeled cardiomyocytes by hypotonic lysis [11] gave a comparable autoradiographic pattern (data not shown). None of these binding proteins became labeled in the absence of the cross-linking agent, DSS (data not shown). In accord with the biochemical competition studies (Fig. 4), [^{125}I]TGF-β2 labeling of these three binding proteins was significantly diminished by excess unlabeled TGF-β2 (Fig. 5A), whereas excess aFGF was non-competitive (Fig. 5B). The relatively low cardiomyocyte TGF-β2 receptor density, the pronounced 'stickiness' of this growth factor [17], and the predominance of [^{125}I]TGF-β2 binding to the high-molecular weight protein obviated absolutely clear 'background' lanes with excess unlabeled ligand.

4. DISCUSSION

TGF-β exerts anti-ischemic cardioprotection by mechanisms postulated to involve effects on heart muscle [5,6]. Although cardiac muscle contains and synthesizes TGF-β [3], the potential of the mammalian cardiomyocyte to express specific cell surface TGF-β receptors has not been rigorously and quantitatively examined. The present work provides several lines of direct biochemical evidence demonstrating that the neonatal rat cardiomyocyte has a population of specific, high-affinity sarcolemmal binding proteins for TGF-β2, one of the major mammalian isoforms [1,2]. The rapidity, saturability, ligand-selectivity, and reversibility of cardiomyocyte TGF-β2 specific binding are all consistent with a receptor-mediated process. Both Scatchard anal-

Table II

Displacement of specifically bound cardiomyocyte [^{125}I]TGF-β2

[^{125}I]TGF-β2 (pM)	Displacement (% total [^{125}I]TGF-β2 specifically bound)			
	0 min	10 min	30 min	60 min
4	0	48.2	63.5	91.8
20	0	87.1	98.5	100.0
40	0	84.3	97.4	100.0

Data are mean % loss of specifically bound cardiomyocyte [^{125}I]TGF-β2 after the labeled cells were incubated with a 1,000-fold molar excess of non-radioactive TGF-β2 for the indicated time periods. Values are averages of three independent experiments; the range about each mean was <10%.

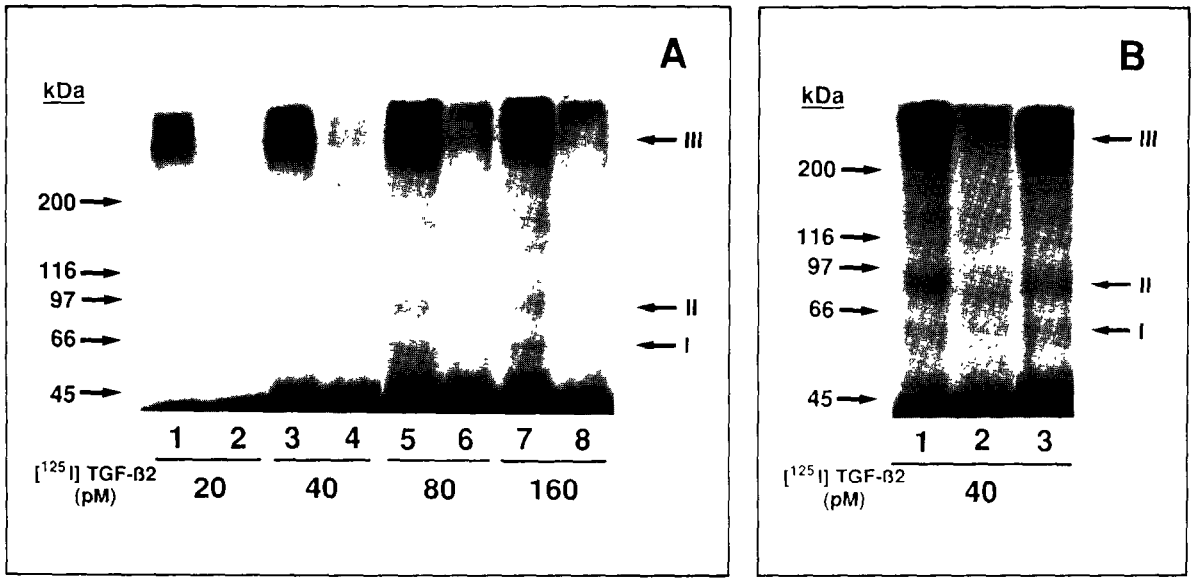

Fig. 5. Affinity labeling of cardiomyocyte TGF-β2 binding proteins. (A) Cardiomyocytes were cross-linked to increasing concentrations of [^{125}I]TGF-β2 in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of a 1,000-fold molar excess of unlabeled ligand. (B) Cardiomyocytes were cross-linked to 40 pM [^{125}I]TGF-β2 in the absence (lane 1) or presence of either a 1,000-fold molar excess of unlabeled TGF-β2 (lane 2) or a 250-fold molar excess of unlabeled aFGF (lane 3). Solubilized cell extracts were subjected to SDS-PAGE on 7% gels, and the dried gels were analyzed by autoradiography. Molecular mass markers are indicated in kDa to the left of each gel series, the locations of putative binding protein types I, II, and III are indicated to the right

ysis [16] and computer-assisted non-linear least-squares iterative fitting [14] demonstrated that the TGF-β2 binding proteins behave as a single class of high-affinity (apparent $K_d \leq 26$ pM) receptors with ~5,000 binding sites/cardiomyocyte and an apparent B_{max} of ~9 fmol/10^6 cells.

While this is the first study to identify and characterize the cardiomyocyte TGF-β2 receptor population, many other cell types bind TGF-β isoforms with apparent K_d's in the pM range [1]. As few as 600 and as many as 8×10^4 TGF-β binding sites/cell have been reported, the highest affinity receptors generally present at the lowest densities [1,2]. Thus, the cardiomyocyte TGF-β2 receptor population has a capacity and affinity comparable to TGF-β receptors in non-muscle cells, albeit toward the lower end of the density range. Although binding characteristics among the TGF-β isoforms in a given cell type may differ, the results of our competition experiments (Fig. 4) suggest either that distinct cardiomyocyte TGF-β1 and -β3 receptor(s) exist or that the cardiomyocyte expresses one population of TGF-β binding proteins having markedly lower affinities for TGF-β1 and -β3 than for -β2.

Chemical cross-linking of [^{125}I]TGF-β2 to cardiomyocytes allowed provisional molecular identification of three discrete TGF-β2 binding proteins. The affinity-labeled cardiomyocyte complexes resemble on SDS-PAGE the type I (~55 kDa), type II (~70–85 kDa), and type III proteoglycan (~250–350 kDa) binding proteins detected on many cell types [1,19]. The type I and type II binding proteins act as the signalling receptor in a complex with ligand, whereas the role of the proteoglycan is a matter of current debate [2]. In the cardiomyocyte, apparently more [^{125}I]TGF-β2 became associated with the proteoglycan than with the type I and II binding proteins (Fig. 5), as has been observed in some other cell types [18]. The presence of high-affinity TGF-β2 receptors on the mammalian cardiomyocyte makes it tempting to speculate that this cytokine family may modulate cardiomyocyte physiology by as yet unknown receptor-mediated pathways of signal transduction, some of which may be cardioprotective.

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