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Mutagenesis analysis of the membrane-proximal ligand binding site of the TGF-β receptor type III extracellular domain

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Abstract There are two TGF-β binding subdomains in the extracellular domain of receptor type III (proximal and distal in relation to the transmembrane domain). Here we present an extension of our analysis of the proximal binding site of receptor type III. Due to the original deletion mutagenesis strategy, our proximal binding site contained 19 amino acids from the N-terminal part of the receptor. By deleting these, we demonstrated that they did not contribute to the binding ability of the proximal binding site. We also produced a soluble, secreted form of the proximal binding site and demonstrated that it was able to bind TGF-β. Finally, we analyzed the role of the three asparagine residues (580, 591, 595) that are located in the region of the receptor that is necessary for expression of a functional proximal binding site, and found that mutation of these residues individually to alanine did not affect ligand binding.

Key words: Transforming growth factor-β receptor type III; Betaglycan; Mutagenesis

1. Introduction

Transforming growth factor β (TGF-β) is a member of a superfamily of growth and differentiation factors that include activins, inhibins, the bone morphogenetic proteins and related morphogenetic peptides. TGF-βs (1–3 in mammals) are closely related secreted peptides which regulate extracellular matrix formation, cell growth and differentiation, morphogenesis and immune responses [1–6]. Most cells display three types of cell surface receptors which can bind and be chemically cross-linked to TGF-β. Those three TGF-β receptors are termed receptor type I (53 kDa), type II (73 kDa) and type III (280 kDa) and can be divided into the signaling receptors (type I and type II) and non-signaling receptors (type III and the related receptor endoglin) [1,2,7–12]. Receptor type III is thought to act as an enhancer of TGF-β access to the signaling receptors [13,14] but not to signal in itself.

The receptors type I and type II seem to be directly involved in signal transduction since the loss of expression of either the type I or the type II receptors results in the loss of response to the growth-inhibitory effect of TGF-β in tumor cell lines [3,10,15–17] or in mutagenized mink lung epithelial cells [18–21]. TGF-β receptors type I and type II are related to each other [22,23]. Their cytoplasmic domains contain serine/threonine kinase activity suggesting that they mediate a phosphorylation-dependent transduction of signal [11,23–25]. Receptor type I is a 53 kDa transmembrane protein which requires the presence of receptor type II in order to bind TGF-β [11,20,24]. On the other hand, receptor type II is a 73 kDa protein that can independently bind TGF-β [23] but requires the presence of receptor type I for signaling [11].

Receptor type III is coexpressed with receptor type I and type II in various cell types and in most cases is the major TGF-β binding molecule on the cell surface [3,10,26,27]. It exists as a proteoglycan with heparin and chondroitin sulfate chains; however, TGF-β can bind to the core of the receptor in the absence of glycosaminoglycan chains [28,29]. Receptor type III is a transmembrane protein with a short cytoplasmic domain showing no apparent signaling motif [30,31,32]. The absence of an obvious signaling domain, together with the fact that various TGF-β responsive cells lack the type III receptor [10,33,34] has led to the proposal that the type III receptor does not participate directly in TGF-β signal transduction. However, receptors type I and II bind TGF-β1 and TGF-β3 with higher affinity than TGF-β2 [7,10,35] and this difference is more pronounced in cells that lack the receptor type III [33,35]. This led to the suggestion that receptor type III might modify the responsiveness of cells to various isoforms of TGF-β. More recently results which demonstrated a direct association between the type III and signaling receptors provided strong support for the role of the receptor type III in delivering TGF-β to the signaling receptors [13,14]). It has been shown that when receptor type III is overexpressed in transfected cells, it increases the affinity of receptor type II for TGF-β2 [13]. However, when expressed in the cell media as a secreted form, the extracellular domain of receptor type III decreases the labeling of receptor type II and type I and reduces the magnitude of the cell's response to TGF-β as if it was sequestrating TGF-β [36].

We have previously reported the analysis of a number of deletion mutants of the TGF-β receptor type III. The shortest binding mutant (Δ44-575) defined a ligand binding region in the 210 amino acids of the extracellular domain which flank the transmembrane domain [37]. Another group also showed the existence of a binding site in this region using *E. coli* expression of fragments of the receptor type III [38]. An approach similar to ours was used by Lopez-Casillas et al. and they demonstrated the existence of a TGF-β binding domain at the N-terminal part of the extracellular domain [36]. Two TGF-β binding domains have thus been identified in receptor type III. The first lies at the N-terminus of the extracellular domain [36]. The other lies at the C-terminal end of the extracellular domain

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Abbreviations: TGF-β, transforming growth factor-β; GAG, glycosaminoglycan.

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in a juxtamembrane position [37,38]. These domains are referred to here as the distal and proximal binding sites respectively with respect to their location relative to the transmembrane domain. The significance of the presence of the two TGF-β binding sites in receptor type III is unclear at present. However, when expressed as secreted proteins both the distal [36] and the proximal [38] binding site are able to modulate TGF-β access to the cells.

Here, we have extended our analysis of the proximal binding site of receptor type III. In order to confirm that the proximal binding site is independent from N-terminal sequences (i.e. from the distal binding site), we have deleted the 19 N-terminal amino acids from mutant Δ44-575. We show that this mutant (Δ26-575) binds TGF-β, thus ruling out the possible contribution of N-terminal sequences to the binding ability of the proximal binding site. Moreover, we have produced a secreted version of mutant Δ44-575 which is able to bind TGF-β indicating that the proximal binding site does not require the cell membrane or membrane proteins to bind TGF-β. We have also performed site directed mutagenesis on mutant Δ44-575 to explore the role of putative *N*-glycosylation sites in a segment (aa 576–596) whose deletion negates expression of an active TGF-β binding site. These results indicate that asparagine-591 is normally glycosylated, but that this glycosylation event is not required for the expression or binding function of the proximal binding site.

2. Materials and methods

2.1. Vector construction

A vector coding for a secreted version of Δ44-575 (Δ44-575sv) was produced by replacing amino acid-783 (a glycine which lies 3 aa upstream the transmembrane domain) by an opal stop codon. Primer RTB3 2642:stop (5′-CCGA.A.TTCTCA.A.^{2665}TGGA.A.A.A.TCTGTGGAGG^{2648}-3′; numbers refer to the numbered sequence published by Lin et al. [23]) which bears the glycine to stop mutation was used along with primer Xba5.1(3) (5′-CCTCTAGA^{24}A.A.GCGGGCTGCTGTCCTTC^{43}-3′ located in the 5′ leader) to PCR amplify the coding region of Δ44-575. The PCR fragment was gel purified, phosphorylated and sub-cloned blunt-ended at the *Eco*RV site of pCDNA3.

A myc epitope was added to mutants Δ44-575 and Δ44-596; these are, respectively, the shortest binding mutant and the longest non-binding mutant from our previous work [37]. Four primers were used for this cloning. Primer Xba5.1(3) is located in the 5′ leader of receptor type III cDNA. Primer R3L (5′-TCGA.A.TTCC^{2990}GGGCGTCTGTCTGTCCAC^{2882}-3′) is located in the 3′ non-translated sequence of receptor type III cDNA. Primer Myc.1 is a chimeric primer pointing toward the 5′-end of the cDNA and linking the sequence for amino acids 25–30 of receptor type III to the sequence for amino acids EQKLI (the N-terminal portion of the Myc epitope) (5′-ATCAGCTTCTGTTCCTCGC^{408}GGGTGCTGGGCTCTGGA^{391}-3′). Primer Myc.2 points toward R3L and links amino acids SEEDLL (the C-terminal portion of the Myc epitope) to amino acids 31 to 36 of receptor type III (5′-CTCCGAGGA.A.GATCTGCTG^{409}TGTGA.A.CTGTCACCA.A.T^{426}-3′). Primer Myc.1 was phophorylated and used along with Xba5.1(3) to PCR amplify the 5′ portion of the cDNA (410 bp) with Vent DNA polymerase (New England Biolabs). Primer Myc.2 was used along with R3L to PCR amplify the 3′ portion of the cDNA (2518 bp) with Vent DNA polymerase. Both fragments were ligated together, gel purified to isolate the expected 2928 bp band which was then phosphorylated and cloned into *Eco*RV-digested and dephosphorylated pCDNA3 vector.

Asparagine to alanine mutants were produced by USE mutagenesis (Pharmacia) using pCDNA3-(Δ44-575) as the parent vector. The selection primer was designed to mutate the unique *Kpn*I site of the multiple cloning site of pcDNA3(Δ44-575) to a unique *Nhe*I site (Primer KtoN: 5′-CCGAGCTCGCTAGCA.A.GCTTGGGTC-3′). Primer RT3-N580 (5′-G^{2068}CCACTGGGAGCCCTCAGCTGC^{2047}-3′), RT3-N591 (5′-G^{2101}A.A.GGTAGCAGCTCCATCGAGC^{2080}-3′) and RT3-N595 (5′-C^{2113}AGCTCCATGGCGA.A.GGTAGC^{2093}-3&prim e;) were used to produce Δ44-575N580A, Δ44-575N591A and Δ44-575N595A, respectively.

Δ26-575(Myc) was produced by USE mutagenesis using pcDNA3(Δ44-575) as the parent vector, KtoN as the selection primer and M6Myc-20 (5′-T^{2058}TCCTCAGCTGCCG^{2045}CAGATCTTCCTCGGAGATCAGCTTCTGTTCT^{394}GGACCGGCGGTGG^{381}-3′) as the mutagenic primer. This primer loops out the sequence corresponding to amino acids 26–43 of receptor type III and replaces it with the Myc epitope (EQKLISEEDL).

The sequence of every mutant was confirmed by dideoxy nucleotide sequencing.

2.2. Cell culture and transfection procedure

Cos-1 cells were grown in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Cells were transiently transfected according to the procedure of Chen and Okayama [39]. Transfections were performed using 20 μg of expression vector and 5×10^5 cells per 6-well plate. Binding of TGF-β to the mutant receptors was monitored 48 h after transfection by a ligand binding and cross-linking assay.

2.3. Binding assay

The binding and cross-linking assays were performed on transfected cells as previously described [40] using TGF-β1 (supplied by Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, WA) or TGF-β2 (supplied by Celtrix Laboratories Inc., Palo Alto, CA) which was iodinated as described in Philip and O'Connor-McCourt [41]. For the cross-linking assay which was performed on cell media, the following procedure was used. 48 h after transfection, cell media was harvested, buffered with 50 mM Na2HPO4 pH7.4 and incubated with 200 pM of [^{125}I]TGF-β2 (with or without 20 nM unlabeled TGF-β2) for 3 h at 4°C. Cross-linking was performed for 2 min at 4°C by addition of 1/10 volume of 10 mM bis sulfosuccinimidyl suberate (BS3) and was stopped by addition of 1/5 volume of 625 μM glycine. Cross-linked receptors were analyzed by polyacrylamide gel electrophoresis (under reducing conditions) and autoradiography.

The expression of the myc-tagged mutants was confirmed by Western blotting using antibody 9E10 which is directed against the myc epitope [42].

3. Results and discussion

3.1. The proximal binding site of receptor type III binds TGF-β independently of N-terminal sequences

The TGF-β binding site of receptor type III has been mapped independently by three laboratories to two different subdomains of the extracellular domain (Fig. 1). One site has been mapped proximal to the transmembrane domain by Fukushima et al. [38] and our group [37]. This region shares distant homology with a subdomain of uromodulin and other proteins [43] (Fig. 1). Another TGF-β binding site was mapped to the N-terminus end of the extracellular domain of the receptor type III by López-Casillas et al. [36]. This region shows homology to endoglin, a TGF-β binding membrane protein that is expressed predominately on endothelial cells [26]. Although it was initially difficult to reconcile the results of Fukushima et al. [38] and ourselves [37] with those of Lopez-Casillas et al. [36], it has since been confirmed in three different laboratories that the mutants which contain the N-terminal site as identified by Lopez-Casillas et al. [36] and the mutants which contain the C-terminal site as identified by our group [37] are all able to bind TGF-β (F. Lopez-Casillas and J. Massagué, personal communication; and K. Myazono, personal communication; M.-C. Pepin, data not shown).

It should be noted, however, that, owing to the deletion

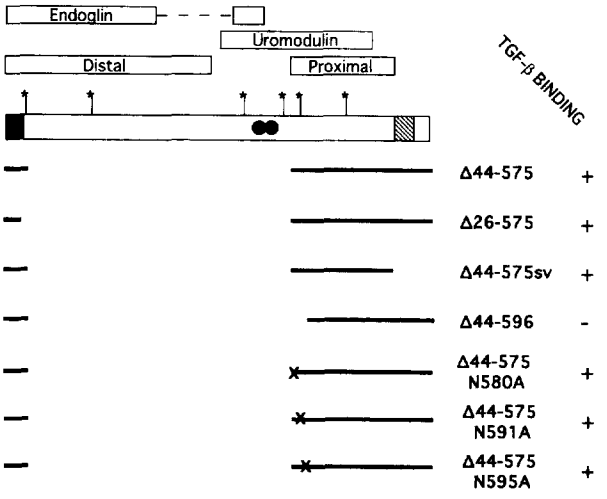

Fig. 1. Schematic representation of TGF-β receptor type III and various mutants (produced during this work) with a summary of their TGF-β binding ability. Regions of homology with endoglin and uromodulin are shown by overlying boxes as well as the location of the proximal and distal TGF-β binding sites. Black box = signal peptide; hatched box = transmembrane domain; black dots = glycosaminoglycan attachment chains; stars = putative *N*-glycosylation sites.

mutagenesis strategy that we used originally [37], there are 19 amino acids from the N-terminal part of the receptor that are present in mutant Δ44-575 (i.e. our shortest binding mutant which defined the proximal binding site [37]). In order to rule out the possibility that these amino acids from the distal binding site might be contributing to the binding ability of mutant Δ44-575, we created mutant Δ26-575 by deleting amino acids 26–43 from mutant Δ44-575 and replacing them with a myc epitope. COS-1 cells were transfected with the expression vector for mutant Δ26-575, and also with mutant Δ44-575 and mutant Δ44-596 as positive and negative controls. Cells were affinity-labeled with iodinated TGF-β, and the cell extracts were separated by electrophoresis and revealed by autoradiography. Fig. 2 shows the labeling patterns that were obtained. As previously reported, mutant Δ44-575 appears as a doublet centered around 55 kDa along with higher molecular weight species which are thought to be cross-linked multimers of mutant Δ44-575. Also as expected, transfection of cells with mutant Δ44-596 did not yield any labeling with [^{125}I]TGF-β2 (even though mutant Δ44-596 is expressed; see below). Mutant Δ26-575 is clearly labeled by [^{125}I]TGF-β2 (Fig. 2) indicating that the N-terminal sequence (aa 26 to 43) does not make an essential contribution to the binding ability of mutant Δ44-575, i.e. the proximal binding site. That the proximal site binds independently from N-terminal sequences is supported by the results of Fukushima et al. [38] since their bacterially produced fusion protein which included amino acids 543–769 of receptor type III behaved as a TGF-β binding protein. The labeling pattern of mutant Δ26-575 is similar to but distinct from that of mutant Δ44-575. The oligomeric forms of the receptor are present as observed with mutant Δ44-575. However, whereas mutant Δ44-575 appears as a doublet, mutant Δ26-575 migrates as a single band with an apparent molecular weight approximately the same as that of the fastest migrating of the two Δ44-575 bands. This result may be explained by heterogeneity of glycosylation at the predicted *N*-glycosylation site, asparagine-37. If this site is glycosylated in only half of the molecules of mutant Δ44-575, this mutant should appear as doublet and this was observed. Since asparagine-37 is deleted in mutant Δ26-575, this source of heterogeneity would be lost and mutant Δ26-575 should appear as a single band co-migrating with the fastest migrating form of mutant Δ44-575, as was observed.

3.2. *The proximal binding site of receptor type III binds TGF-β independently of membrane anchorage*

In order to confirm that the proximal site is a bona fide TGF-β binding subdomain independent from accessory structures or proteins on the cell surface, we produced a secreted version of mutant Δ44-575 and analyzed its ability to bind in a cell-free environment. Mutant Δ44-575sv (for soluble version) was created by inserting a stop codon at position 783, 3 amino acids upstream of the transmembrane domain. When media from transfected cells were affinity labeled, a TGF-β binding protein of the expected size was observed for cells transfected with mutant Δ44-575sv, but not with mutant Δ44-575 or mock-transfected cells (Fig. 3). These results demonstrate that the proximal binding site is functional when secreted from cells in a soluble form.

3.3. *The non-binding mutant (Δ44-596) is expressed*

In our previous work [37], we observed that deletion mutant Δ44-596 could not be detected by affinity-labeling, suggesting that this mutant is unable to bind TGF-β or that it is not expressed at the cell surface. To verify the expression of mutant

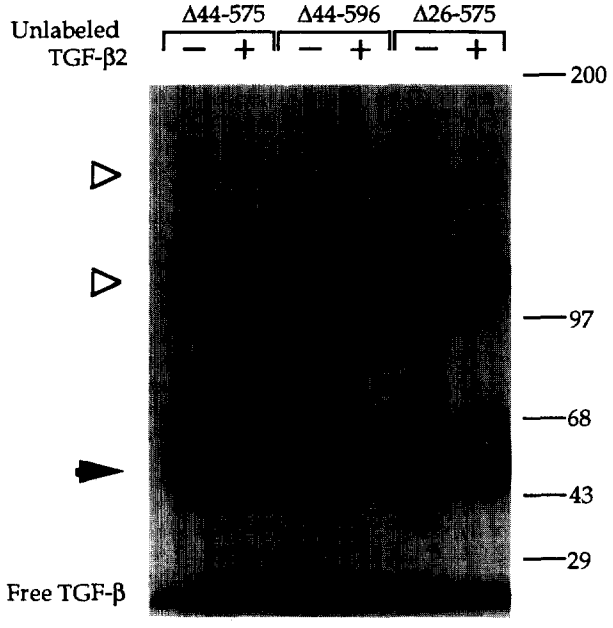

Fig. 2. TGF-β2 affinity-labeling of Cos-1 cells transiently transfected with expression vectors for deletion mutants of TGF-β receptor type III. Cell transfection, affinity-labeling and gel electrophoresis were done as described in section 2. The presence or absence of unlabeled competitor is shown by the + or – signs. The filled arrow headpoints to the monomeric form of the mutants whereas the empty arrow heads point toward labeled species of higher apparent molecular weight that are thought to be oligomers of the receptor mutants. Molecular weight markers in kDa are shown on the right hand site.

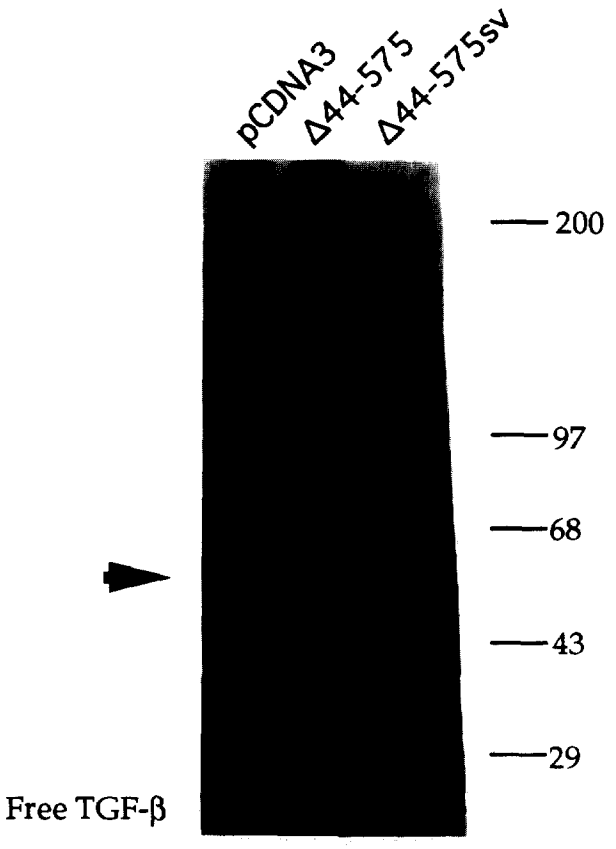

Fig. 3. TGF-β2 affinity-labeling performed on culture media of cells transfected with an expression vector for a secreted form of Δ44-575 (Δ44-575sv). Cell transfection, affinity-labeling and gel electrophoresis were as described in section 2. The arrowhead points to the labeled soluble form of the proximal binding site. The molecular weight markers are shown on the right-hand side.

Δ44-596, we added a c-myc epitope on mutant Δ44-596, and also on mutant Δ44-575 as a control. When transfected in COS-1 cells, both constructs yielded bands around the expected molecular weight that could be detected using the anti-myc monoclonal antibody 9E10 [42] on Western blot (Fig. 4). Mutant Δ44-575 appears as a doublet of 41 and 44 kDa whereas its predicted molecular weight is 32.8 kDa. Mutant Δ44-596 shows up as a triplet of 39, 42 and 46 kDa whereas its predicted molecular weight is 30.5 kDa. As discussed above for mutant Δ44-575, the appearance of the doublet and triplet is likely due to heterogeneity of glycosylation. The difference in molecular weight between the fastest migrating band of each mutant (41 kDa–39 kDa) is consistent with the difference expected from their predicted molecular weights (32.8 – 30.5). The higher apparent molecular weight of the affinity labeled form of mutant Δ44-575 as compared to that of the Western blot (Fig. 4) is due to the presence of a cross-linked monomer of TGF-β (12.5 kDa).

In order to demonstrate that the myc tagged Δ44-596 is not only expressed, but is also localized at the cell surface, we attempted cell surface labeling, immunocytochemistry and glycosylation pattern analysis, but none of these experimental approaches gave conclusive results. Deglycosylation experiments showed that the majority of the expressed Δ44-596 and Δ44-575 mutants was sensitive to endoH (data not shown). This demonstrates that in our transfection experiments, the majority of the expressed proteins have immature glycosylation chains suggesting that they are retained inside the cells. However, it is evident that some of mutant Δ44-575 reaches the cell surface since it can be labeled by TGF-β.

3.4. *Analysis of potential glycosylation sites in the 576–596 region of mutant Δ44-575*

Since mutant Δ44-575 binds TGF-β and mutant Δ44-596 does not, it appears that amino acids 576–596 are necessary for the expression of a functional proximal binding site at the cell surface. In order to identify important residues in these 20 amino acids, we independently mutated to alanines three asparagines located at position 580, 591 and 595 of mutant Δ44-575 (Δ44-575N580A, Δ44-575N591A, Δ44-575N595A). The mutant vectors were transiently transfected in COS-1 cells that were affinity-labeled using [^{125}I]TGF-β2. It can be seen in Fig. 5 that the three mutants bind TGF-β indicating that these amino acids are not critical for the transport, folding and binding function of the proximal binding site. Moreover, it can be seen that all the labeled species observed with mutant Δ44-575N591A have a lower apparent molecular weight than those of mutants Δ44-575N585A and Δ44-575N595A (which both co-migrate with mutant Δ44-575; data not shown). This strongly suggests that asparagine-591 is glycosylated, consistent with predictions made from analysis of the sequence [31,32]. The glycosylation of this site is not critical for TGF-β binding, a finding consistent with the fact that the proximal binding site produced in bacteria behaves as a TGF-β binding protein [38].

We conclude from our work that the domain covering amino acids 575–783 of TGF-β receptor type III is a bone fida TGF-β binding subdomain which is active independent of N-terminal sequences and anchorage to the cell membrane. Whether the distal and proximal binding sites of TGF-β receptor type III have different independent functions or act in concert is unknown. Work is currently in progress to overexpress the

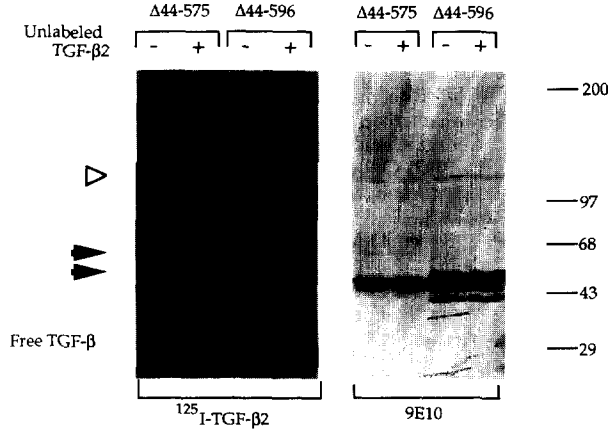

Fig. 4. Analysis of expression of the non-binding mutant Δ44-596. Cos-1 cells were transfected with the expression vector for Δ44-575(Myc) or Δ44-596(Myc) and affinity-labeled with [^{125}I]TGF-β. Cell extracts were submitted to gel electrophoresis and transferred to nitrocellulose. The membrane was submitted to autoradiography ([^{125}I]TGF-β2, left panel) and revealed with a monoclonal antibody against the Myc epitope (9E10), right panel. Unlabeled receptors migrate faster than the labeled ones because the latter are cross-linked to TGF-β monomers (12.5 kDa). Arrow heads and molecular weight markers are as in Fig. 2.

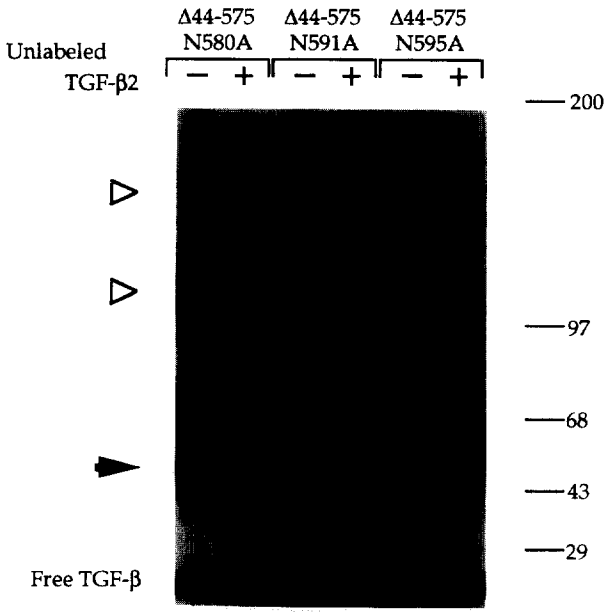

Fig. 5 TGF-β2 affinity-labeling of Cos-1 cells transfected with expression vectors for mutants of Δ44-575 carrying asparagine to alanine substitutions. Cell transfection, affinity-labeling and gel electrophoresis were as described in section 2. Arrowheads and molecular weight markers are as in Fig. 2.

secreted version of mutant Δ44-575 to study its binding characteristics in more detail.

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