Neurotrophin Binding to Human α_2-Macroglobulin under Apparent Equilibrium Conditions†

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ABSTRACT: α_2-Macroglobulin (α_2M) is a broad-spectrum proteinase inhibitor and a carrier of certain growth factors. The purpose of this investigation was to characterize the interaction of α_2M with nerve growth factor-β (NGF-β), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and ciliary neurotrophic factor (CNTF) under apparent equilibrium conditions. Binding in solution was assessed using the cross-linking agent bis(sulfosuccinimidyl) suberate (BS^3). Noncovalent binding of NGF-β, NT-3, NT-4, and BDNF to native α_2M and α_2M–methylamine (a conformationally modified form of α_2M that is recognized by the α_2M receptor) reached apparent equilibrium in less than 20 min at 37 °C. Apparent K_D values for the binding of NT-4, NGF-β, NT-3, and BDNF to α_2M–methylamine were 61, 110, 120, and 150 nM, respectively. Native α_2M bound all four neurotrophins with decreased affinity. Unlabeled NGF-β competed with the radioiodinated neurotrophins for binding to immobilized α_2M–methylamine. The K_I for unlabeled NGF-β was 120 nM, in good agreement with the apparent K_D determined by the BS^3 method. The number of NGF-β binding sites per immobilized α_2M–methylamine was 1.0. CNTF bound minimally, if at all, to native α_2M and α_2M–methylamine as determined using a number of techniques. The extent of binding was insufficient for the determination of an affinity constant. The studies presented here indicate that all four neurotrophins bind with similar affinity to the same site in α_2M which is available primarily after the α_2M undergoes conformational change. Interaction of neurotrophins with α_2M may be important in regulating neurotrophin activity and/or availability.

Neurotrophic factors promote neuronal survival and differentiation during development, provide for the functional maintenance of neurons during normal homeostasis, and play vital roles in neuronal regeneration and responses to injury (Hefti *et al.*, 1993). Nerve growth factor-β (NGF-β),[1] brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are structurally and functionally related proteins that comprise the family of neurotrophic factors called neurotrophins [reviewed in Eide *et al.* (1993) and Glass and Yancopoulos (1993)]. The neurotrophins are small ($M_r \sim 26\,000$), basic homodimers with an overall sequence identity of ~55% (Radziejewski *et al.*, 1992; Narhi *et al.*, 1993; Glass & Yancopoulos, 1993; Fandl *et al.*, 1994). A second class of neurotrophic factors is represented by ciliary neurotrophic factor (CNTF). CNTF is a small ($M_r \sim 24\,000$), acidic protein that is structurally distinct from the neurotrophins (Lin *et al.*, 1989; Manthorpe *et al.*, 1993).

The neurotrophins and CNTF mediate their biological effects via different receptors (Ip & Yancopoulos, 1993). The Trk family of tyrosine kinase receptors forms a necessary component of functional neurotrophin receptors. NGF-β initiates intracellular signalling through TrkA; BDNF and NT-4, via TrkB; and NT-3, preferentially via TrkC [reviewed in Chao (1992), Meakin and Shooter (1992), Raffioni *et al.* (1993), and Ip and Yancopoulos (1993)]. All of the neurotrophins also bind with comparable affinity ($K_D \sim 1$ nM) to the p75 NGF receptor ($p75^{NGFR}$). $p75^{NGFR}$ has no known signalling capacity; however, this receptor may signal indirectly by associating with intracellular serine/threonine kinases (Volonte *et al.*, 1993). Soluble forms of $p75^{NGFR}$ have been described; the soluble receptors apparently antagonize neuronal regeneration *in vivo* (DiStefano & Johnson, 1988; DiStefano *et al.*, 1993).

In contrast with the neurotrophins, CNTF functions through a multicomponent receptor whose subunits are members of the cytokine receptor superfamily (Davis *et al.*, 1991; Ip & Yancopoulos, 1993). Soluble components of the CNTF receptor promote CNTF action and may confer CNTF responsiveness in normally unresponsive cells (Davis *et al.*, 1993a,b).

Factors that regulate the extracellular availability and distribution of neurotrophic factors remain largely uncharacterized. The proteinase inhibitor α_2-macroglobulin (α_2M) binds NGF-β reversibly and noncovalently (Ronne *et al.*, 1979; Koo & Stach, 1989; Koo & Liebl, 1992; Crookston *et al.*, 1994). A brief report published while this work was in progress suggested that α_2M may also bind NT-3 and CNTF; however, the affinities of these interactions were not determined (Liebl & Koo, 1993a). The neurotrophin-binding activity of α_2M may be responsible for the observed decrease in NGF-β activity *in vitro* in the presence of α_2M (Koo & Liebl, 1992; Liebl & Koo, 1993b). It is also possible that α_2M inhibits the activity of NGF-β by competing with the growth factor for binding to Trk A (Koo & Qiu, 1994).

α_2M is a large glycoprotein (M_r 718 000) that functions as a proteinase inhibitor and as a carrier of certain cytokines

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[1] Abbreviations: NGF-β, nerve growth factor-β; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; CNTF, ciliary neurotrophic factor; α_2M, α_2-macroglobulin; $p75^{NGFR}$, p75 NGF receptor; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; BS^3, bis(sulfosuccinimidyl) suberate.

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[reviewed in Borth (1992) and Gonias (1992)]. α_2M is present in the plasma at high concentration (2–4 μM) and in the extracellular spaces. Proteinases that react with α_2M cause a major conformational change in the proteinase inhibitor; this conformational change traps the proteinase in a nondissociable complex. α_2M conformational change also exposes β-cysteinyl-γ-glutamyl thiol ester bonds (one per α_2M subunit). The exposed thiol esters either react with nucleophilic side chains in the proteinase, covalently stabilizing the α_2M–proteinase complex, or are nonproductively hydrolyzed by H_2O. Thiol ester cleavage yields the only free cysteine residues in α_2M.

Small primary amines, such as methylamine, cleave the α_2M thiol esters in the absence of proteinase, inducing α_2M conformational change that is equivalent to the change caused by proteinases (Gonias *et al.*, 1982). α_2M–methylamine and α_2M–proteinase complexes lack further proteinase-binding activity and are recognized by the cellular receptor, low density lipoprotein receptor-related protein (LRP). Native α_2M is not LRP-recognized. For these reasons, α_2M–methylamine is frequently studied as a model of conformationally transformed α_2M species, collectively referred to as activated α_2M (α_2M*).

Binding of growth factors, such as NGF-β, to α_2M occurs by a mechanism that is distinct from proteinase trapping (Borth, 1992; Gonias, 1992; Crookston *et al.*, 1994). Either conformation of α_2M (native α_2M or α_2M*) may be involved, and the α_2M structure is not significantly changed by the interaction. Association of growth factors with α_2M (or α_2M*) is initially reversible and noncovalent (Crookston *et al.*, 1994). For the cytokines studied thus far, the reversible binding step reaches apparent equilibrium relatively quickly (about 10 min). Noncovalent growth factor–α_2M* complexes may be slowly converted into covalent complexes as a result of thiol–disulfide exchange.

The fate of growth factors that bind to α_2M *in vivo* is dependent on the α_2M conformation. If the α_2M is activated (LRP-recognized), then the growth factor is targeted to cells that express LRP; this process may result in growth factor clearance or regulate growth factor availability to different cell types (Danielpour & Sporn, 1990; LaMarre *et al.*, 1991; Stouffer *et al.*, 1993; Gonias *et al.*, 1994). By contrast, growth factors that are bound to native α_2M are restricted from signalling receptors and protected from clearance pathways or proteolytic degradation (Koo & Stach, 1989; Crookston *et al.*, 1993).

Binding of transforming growth factor-β1 (TGF-β1) and TGF-β2 to α_2M decreases TGF-β activity in some cell culture systems (Danielpour & Sporn, 1990; Hall *et al.*, 1992). In order to predict whether α_2M may regulate the activities of neurotrophic factors by a similar mechanism, binding affinities must be determined. We previously reported apparent equilibrium dissociation constants for the binding of NGF-β to native α_2M and α_2M–methylamine (Crookston *et al.*, 1994). In this investigation, we compared the binding affinities of native α_2M and α_2M* for BDNF, NT-3, NT-4, and CNTF. The results suggest that all of the neurotrophins bind α_2M. The highest affinity interaction was measured with NT-4. By contrast, binding of CNTF to α_2M was minimal and probably not significant.

EXPERIMENTAL PROCEDURES

Materials. $Na^{125}I$ was from Amersham International, and Enzymobeads were from Bio-Rad. Ninety-six-well Immulon microtiter plates were purchased from Baxter Scientific. Siliconized polypropylene tubes were obtained from National Scientific. Methylamine-HCl, 1,4-dithiothreitol, and bovine serum albumin (BSA) were from Sigma. Bis(sulfosuccinimidyl) suberate (BS^3) was from Pierce. Mouse submaxillary glands were from Pel Freez (Rogers, AZ).

Proteins. Native α_2M was purified from human plasma according to the method of Imber and Pizzo (1981). The preparations were free of conformationally transformed α_2M, as determined by nondenaturing PAGE. α_2M–methylamine was prepared by dialyzing α_2M against 200 mM methylamine-HCl and 50 mM Tris-HCl, pH 8.2, for 16 h at 22 °C, followed by exhaustive dialysis against 20 mM sodium phosphate and 150 mM NaCl (PBS). Reaction of native α_2M with methylamine was confirmed by loss of trypsin-binding activity (>96%). NGF-β was purified from mouse submaxillary glands as described previously (Darling & Shooter, 1984). Human α_2M and murine α_2M bind purified murine NGF-β equivalently (Wolf *et al.*, unpublished results). Recombinant human BDNF, NT-3, NT-4, and CNTF were generously provided by Regeneron Pharmaceuticals (Tarrytown, NY).

Radioiodination. Neurotrophins and CNTF were radioiodinated using $Na^{125}I$ and matrix-bound glucose oxidase/lactoperoxidase (Enzymobeads) according to the manufacturer's instructions. This method permits radioiodination of neurotrophins to high specific activity without loss of biological activity (Escandon *et al.*, 1993). Desalting was performed on Sephadex-G-25 (Pharmacia) equilibrated in 50 mM sodium acetate and 0.5 M NaCl, pH 4.0, containing 1 mg/mL BSA. Greater than 97% of the radioactivity in [^{125}I]neurotrophic factor preparations was precipitable with trichloroacetic acid. Specific activities ranged from 5.0 to 15.0 μCi/μg.

Determination of Apparent Equilibrium Dissociation Constants. Binding of many growth factors to α_2M may be described by the following two-step model:

$$A + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AC \xrightarrow{k_2} AC^* \quad (1)$$

A is unbound α_2M; C is free (unbound) growth factor; AC is reversibly associated (noncovalent) α_2M–growth factor complex; and AC* is irreversibly associated (covalent) α_2M–growth factor complex (Crookston *et al.*, 1994; Gonias *et al.*, 1994). Conversion of AC to AC* occurs via thiol–disulfide interchange. Since only α_2M–methylamine and α_2M–proteinase have free cysteine residues, the covalent stabilization reaction occurs primarily with activated α_2M (α_2M*). The model shown in eq 1 applies for α_2M that is not actively undergoing conformational change (no proteinase or methylamine present in the system). For a series of growth factors studied previously (including NGF-β), we showed that k_2 is small compared with k_{-1} (Crookston *et al.*, 1994). Under these conditions, an apparent K_D for the reversible step of the binding interaction is determined as follows:

$$\frac{[C]}{[AC]} = K_D \frac{1}{[A]} \quad (2)$$

In this investigation, [^{125}I]BDNF (2–4 nM), [^{125}I]NT-3 (2–4 nM), [^{125}I]NT-4 (5–10 nM), or [^{125}I]CNTF (10–20 nM) was incubated with excess native α_2M or α_2M–methylamine (typically 0.06–2.0 μM) in PBS containing 1 mg/mL BSA at 37 °C. At the indicated times, BS^3 (5 mM, final concentration) was added for 1 min at 37 °C. H_2O alone was added to identical control incubations. BS^3-cross-linking reactions were terminated by acidification with HCl. Samples were then denatured in 2% SDS for 30 min at 37 °C

and subjected to SDS–PAGE after Tris-HCl (100 mM final concentration) and glycerol (10%, v/v) were added. Dried gels were subjected to autoradiography. The amounts of free ^{125}I-labeled growth factor and α_2M–growth factor complex (including covalent complex and BS^3-stabilized noncovalent complex) were quantitated by slicing the gels and counting the slices in a γ counter. Covalent ^{125}I-labeled growth factor–α_2M complex (AC*) was determined by analyzing binding in solutions that were not treated with BS^3. Since the subunits in neurotrophin homodimers are not disulfide bonded, recovery of AC and AC* was corrected for homodimer dissociation in SDS (assuming that only one of the two neurotrophin subunits is cross-linked to α_2M). In control experiments, less than 5% of the neurotrophin homodimers were covalently stabilized by BS^3 in the absence of α_2M. To determine apparent K_D values, results were plotted according to the following expression (Crookston *et al.*, 1994):

$$\frac{[C]_e}{[AC]_e} = \left(\frac{K_D}{z}\right)\left(\frac{1}{[A]}\right) + \left(\frac{1}{z} - 1\right) \quad (3)$$

$[C]_e$ is the experimentally detected concentration of free ^{125}I-labeled growth factor. $[AC]_e$ is the experimentally detected concentration of noncovalent ^{125}I-labeled growth factor–α_2M complex (total complex detected with BS^3 minus the amount of covalent complex). [A] is the α_2M concentration (total α_2M and free α_2M are equivalent since α_2M is present in excess). The cross-linking efficiency (z) is a constant ($0 < z < 1$) for each growth factor, α_2M species, and set of experimental conditions (Crookston *et al.*, 1994.). z does not vary as a function of α_2M concentration so that $[AC]_e = z[AC]$. In determining apparent K_D values by this method, it is assumed that (1) each α_2M has one growth factor binding site, (2) all α_2M molecules in a given preparation bind the growth factor with equivalent affinity, and (3) the BS^3 does not significantly alter the equilibrium. The last point was addressed experimentally in a previous study (Crookston *et al.*, 1994). All experiments were performed in quadruplicate. The K_D values were averaged and are presented with the standard error of the mean.

Binding Experiments with Immobilized α_2M.* α_2M–methylamine was immobilized in 96-well microtiter plates as described by Webb *et al.* (1992). Briefly, α_2M–methylamine (100 μg/mL) was incubated in the wells for 2 h at 37 °C. Wells were blocked with Tween-20 (0.1%, v/v) for 2 h at 37 °C and then washed extensively. In control experiments with [^{125}I]α_2M–methylamine, we determined that 158 ± 10 fmol (n = 6) of α_2M–methylamine was immobilized per well.

In experiments, a constant concentration of [^{125}I]neurotrophin was incubated with immobilized α_2M–methylamine in the presence of increasing concentrations of non-radiolabeled NGF-β (10 nM to 3.0 μM). Incubations were conducted in PBS containing 1 mg/mL BSA (PBS/BSA) for 1 h at 37 °C. Wells were then washed three times with ice-cold PBS/BSA. [^{125}I]Neurotrophin, which was bound to the immobilized α_2M–methylamine, was recovered in 0.1 M NaOH and 2% SDS and quantified in a γ counter. Specific radioligand binding was determined, in the absence of unlabeled NGF-β, as the difference between total binding and binding observed in blocked wells without α_2M–methylamine. Specific binding represented greater than 90% of total binding in the experiments presented here. Results were analyzed by plotting the specific binding versus the log of the unlabeled NGF-β concentration. Apparent equilibrium inhibition constants (K_I) were determined according to the equation of Cheng and Prusoff (1973):

$$K_I = \frac{IC_{50}}{1 + \frac{[F]}{K_D}} \quad (4)$$

IC_{50} is the experimentally determined concentration of unlabeled NGF-β that decreases [^{125}I]neurotrophin binding by 50%; [F] is the concentration of free [^{125}I]neurotrophin; and K_D is the apparent equilibrium dissociation constant for [^{125}I]neurotrophin binding to α_2M–methylamine (as determined in BS^3 cross-linking studies).

The maximum number of specific NGF-β-binding sites in wells with immobilized α_2M–methylamine (B_{max}) was determined from the competitive binding experiments with [^{125}I]-NGF-β and unlabeled NGF-β, according to the following expression (DeBlasi *et al.*, 1989):

$$B_{max} = \frac{B_0 IC_{50}}{[F]} \quad (5)$$

B_0 is the specific binding of [^{125}I]NGF-β observed in the absence of unlabeled NGF-β. [F] is the concentration of free [^{125}I]NGF-β in the well. Determination of the B_{max} by this method requires the following assumptions: (1) binding of NGF-β to immobilized α_2M–methylamine is adequately described by a single K_D, and (2) [^{125}I]NGF-β and unlabeled NGF-β bind to immobilized α_2M–methylamine with equal affinity.

Binding of [^{125}I]CNTF and [^{125}I]NT-4 to α_2M as Determined by Nondenaturing PAGE and Autoradiography. [^{125}I]-CNTF and [^{125}I]NT-4 were incubated with α_2M or α_2M–methylamine (0.7 μM) in PBS for 1 h at 37 °C. Samples were then subjected to nondenaturing PAGE (5% slabs) at constant voltage (150 V) for 2 h using the buffer system described by Van Leuven *et al.* (1981). Radioligand binding to α_2M was assessed by autoradiography. During electrophoresis, noncovalent α_2M–growth factor complexes dissociate; the extent of dissociation may be significant and difficult to estimate (Gonias *et al.*, 1994). Therefore, growth factor binding to α_2M, as determined by nondenaturing PAGE, may not be representative of equilibrium binding.

FPLC Analysis of CNTF Binding to α_2M. [^{125}I]CNTF (2 nM) was incubated with native α_2M or α_2M–methylamine (1.1 μM) for 1 h at 37 °C. [^{125}I]CNTF–α_2M complexes were resolved from free [^{125}I]CNTF by chromatography on Superose 6 (flow rate 0.4 mL/min). The extent of binding was determined by the radioactivity co-eluting at an early volume with α_2M. Purified [^{125}I]CNTF–α_2M complexes were subjected to SDS–PAGE (5% slabs) under reducing and nonreducing conditions to assess covalent binding.

RESULTS

Demonstration of Apparent Equilibrium. In initial experiments, [^{125}I]neurotrophin binding to α_2M was examined as a function of time, using the BS^3-stabilization method. Figure 1 shows experiments performed with NT-3. Binding of [^{125}I]-NT-3 to native α_2M and α_2M–methylamine reached maximal levels within 20 min at 37 °C. At apparent equilibrium, less binding of NT-3 to native α_2M was observed, even though equivalent concentrations of native α_2M and α_2M–methylamine (1.0 μM) were present. In experiments with [^{125}I]-NGF-β, [^{125}I]BDNF, and [^{125}I]NT-4, apparent equilibrium was achieved in 20 min or less (results not shown). On the

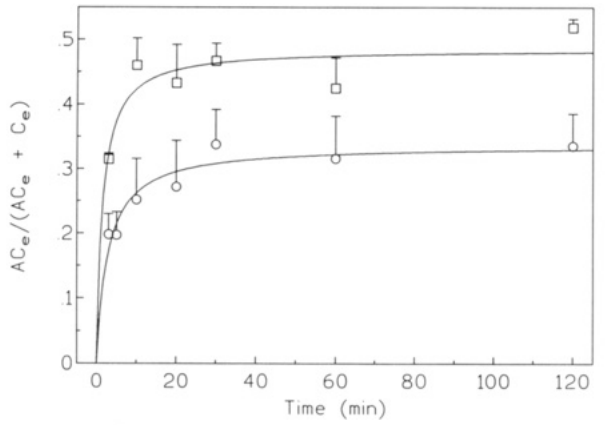

FIGURE 1: [^{125}I]NT-3 binding to α_2M as a function of time. [^{125}I]-NT-3 (5 nM) was incubated with 1.0 μM native α_2M (○) or α_2M–methylamine (□) at 37 °C. Binding was determined by the BS^3 method. The results represent the average ± SEM of three independent experiments with duplicate determinations in each.

Table 1: Apparent Equilibrium Dissociation Constants for Binding of [^{125}I]Neurotrophins to α_2M[a]

neurotrophin	K_D with α_2M–methylamine (nM)	K_D with native α_2M (nM)
NGF-β[b]	110 ± 10	340 ± 60
BDNF	150 ± 10	310 ± 80
NT-3	120 ± 10	340 ± 90
NT-4	61 ± 6	190 ± 20

[a] The data represent the average ± SEM of four independent experiments. [b] Data from Crookston *et al.* (1994).

basis of these results, we decided to incubate each neurotrophin with α_2M for 1 h at 37 °C in order to determine apparent K_D values.

In experiments with [^{125}I]CNTF and native α_2M or α_2M–methylamine, significant binding was not demonstrable using the BS^3-stabilization method. Equivalent results were obtained when the concentration of α_2M (or α_2M–methylamine) was increased to 2.75 μM. These results may be explained if CNTF does not bind to α_2M or if the cross-linking efficiency (z) of the α_2M–CNTF complex is very low.

Determination of Apparent K_D Values. Figure 2 shows representative autoradiographs of [^{125}I]NT-4 binding to different concentrations of α_2M–methylamine in the presence and absence of BS^3. Covalent [^{125}I]NT-4–α_2M complexes migrated primarily in a single low-mobility band. Experimentally detected free [^{125}I]NT-4 ($[C]_e$) migrated near the dye front. In the absence of BS^3, minimal [^{125}I]NT-4–α_2M–methylamine complex was detected. Therefore, the amount of covalent NT-4–α_2M–methylamine complex (stabilized by thiol–disulfide exchange) was small. In control experiments, binding of NT-4 or other [^{125}I]neurotrophins to BSA was not observed, with or without BS^3.

Results of the BS^3-stabilization experiments were analyzed according to eq 3. For each experiment, $[C]_e/[AC]_e$ was plotted against $1/[A]$ to yield an apparent K_D value. Figure 3 presents composites of results obtained with BDNF, NT-3, and NT-4. All plots were linear with correlation coefficients greater than 0.90. The apparent K_D values are presented in Table 1. The affinities of NGF-β, NT-3, and BDNF for α_2M–methylamine were equivalent. NT-4 bound α_2M–methylamine with somewhat higher affinity (61 nM).

Each neurotrophin bound native α_2M with lower affinity than α_2M–methylamine. Once again, the tightest interaction was observed with NT-4. Some native α_2M preparations may contain trace concentrations of minimally modified forms that bind growth factors with higher affinity (LaMarre *et al.*, 1991). We were not able to detect such species in our native α_2M preparations by nondenaturing PAGE; however, if these species were present, they would be expected to decrease the apparent K_D values for the binding of neurotrophins to native α_2M.

Covalent Binding of [^{125}I]Neurotrophins to α_2M. Cross-linking efficiencies (z) were calculated from the y-intercepts of the $[C]_e/[AC]_e$ versus $1/[A]$ plots shown in Figure 3. The z-values are presented in Table 2. With each neurotrophin, the z-value was higher in experiments with α_2M–methylamine compared with native α_2M. The z-values were used to convert $[AC]_e$ into $[AC]$ according to the relationship $[AC]_e = z[AC]$. This calculation permitted an estimation of the total amount of noncovalent α_2M-neurotrophin complex (AC) present in the various incubation mixtures prior to the addition of BS^3. The percentage of the α_2M–neurotrophin complex which was

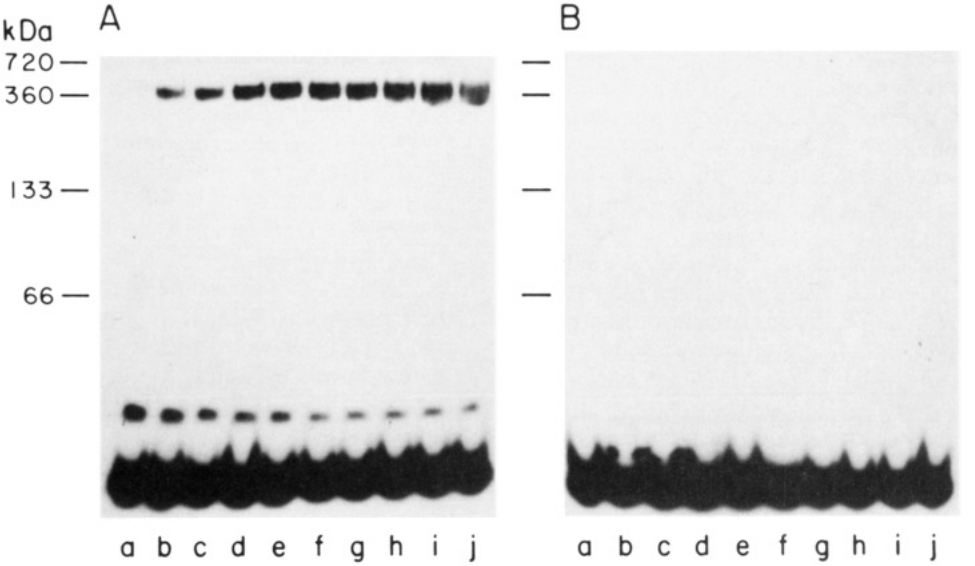

FIGURE 2: Autoradiograph showing [^{125}I]NT-4 binding to α_2M–methylamine. [^{125}I]NT-4 (10 nM) was incubated with α_2M–methylamine at 37 °C. After 1 h, solutions were treated with 5 mM BS^3 (panel A) or H_2O (panel B) for 1 min before SDS–PAGE (no reductant) was performed. The α_2M–methylamine concentrations were as follows: a, 0 nM; b, 31 nM; c, 63 nM; d, 0.13 μM; e, 0.25 μM; f, 0.50 μM; g, 0.75 μM; h, 1.0 μM; i, 1.5 μM; j, 2.0 μM.

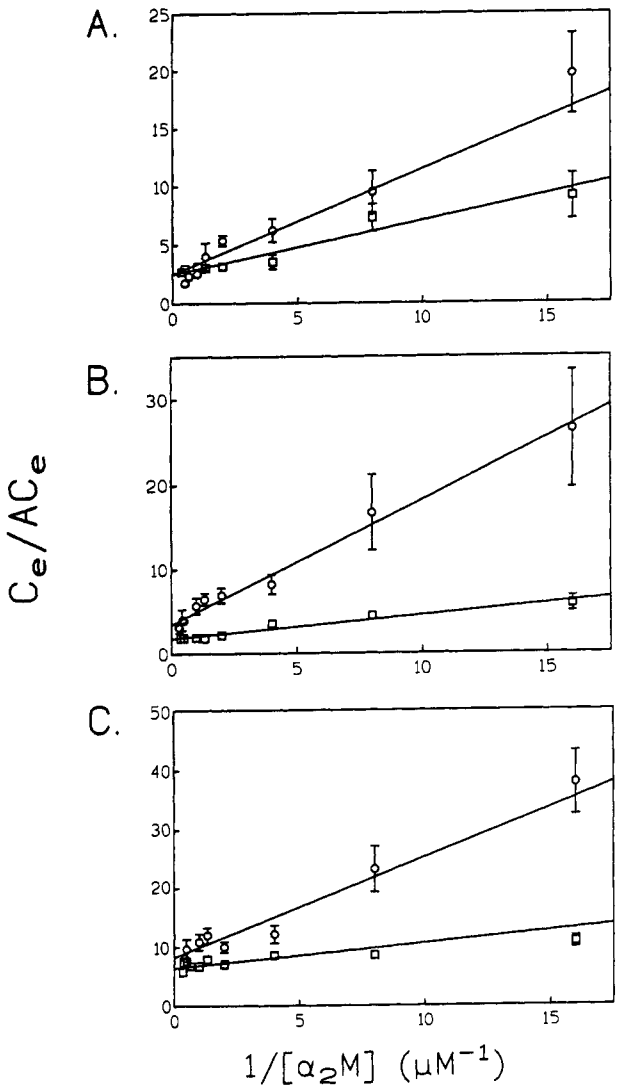

FIGURE 3: Determination of apparent K_D values for [^{125}I]neurotrophin binding to native α_2M and α_2M–methylamine. The results of four independent experiments were combined to generate the presented plots showing binding of [^{125}I]BDNF (panel A), [^{125}I]NT-3 (panel B), and [^{125}I]NT-4 (panel C) to native α_2M (○) and α_2M–methylamine (□). The results were plotted according to eq 3. Error bars represent 1 SEM.

covalently stabilized by thiol–disulfide exchange (prior to the addition of BS^3) was then determined. As shown in Table 2, minimal covalent binding was observed with all of the neurotrophins. The extent of covalent binding was typically higher with α_2M–methylamine than with native α_2M; this was expected since native α_2M does not have free cysteine residues.

Neurotrophin Binding to Immobilized α_2M. Each [^{125}I]-neurotrophin was incubated with immobilized α_2M–methylamine for 1 h at 37 °C. The neurotrophin concentrations and the resulting specific binding levels were as follows: NGF-β, 43 nM, 40 ± 3 fmol; BDNF, 18 nM, 29 ± 6 fmol; NT-3, 23 nM, 27 ± 4 fmol; and NT-4, 9 nM, 44 ± 3 fmol ($n \geq 4$). Unlabeled NGF-β decreased binding of each [^{125}I]neurotrophin to immobilized α_2M–methylamine (Figure 4). The decrease in binding was approximately 80% complete with all of the [^{125}I]neurotrophins. This result indicates that the neurotrophins bind to the same or overlapping sites in activated α_2M.

The concentration of unlabeled NGF-β required to reduce specific binding of each [^{125}I]neurotrophin by 50% (IC_{50}) was 160 nM with [^{125}I]NGF-β, 140 nM with [^{125}I]BDNF, 100 nM with [^{125}I]NT-3, and 170 nM with [^{125}I]NT-4. These IC_{50} values provide an estimate of the binding affinity of non-radiolabeled NGF-β for immobilized α_2M–methylamine. The average IC_{50} (140 nM) is similar to the apparent K_D (110 nM) determined by the BS^3 method using [^{125}I]NGF-β and α_2M–methylamine in solution. The IC_{50} values were corrected to yield K_Is (a more accurate estimate of binding affinity than the IC_{50}). For these calculations, we used the K_D values determined by the BS^3 method (Table 1). The K_I for unlabeled NGF-β (determined by averaging results obtained in experiments with all four radiolabeled neurotrophins) was 120 ± 20 nM.

The B_{max} for NGF-β binding to immobilized α_2M–methylamine (number of NGF-β binding sites) was 150 fmol/well, as determined from the competitive binding experiment with [^{125}I]NGF-β and unlabeled NGF-β. Since the average amount of α_2M–methylamine immobilized in each well was 158 ± 10 fmol, there is approximately 1 NGF-β binding site per immobilized α_2M–methylamine.

[^{125}I]CNTF Binding to α_2M. Since we were not able to demonstrate significant [^{125}I]CNTF binding to α_2M using the BS^3 cross-linking method, a number of other techniques were applied. Figure 5 compares the binding of [^{125}I]CNTF and [^{125}I]NT-4 to α_2M by nondenaturing PAGE and autoradiography. [^{125}I]NT-4 bound to both native α_2M and α_2M–methylamine; greater binding was observed with α_2M–methylamine as expected. By contrast, no binding was observed when [^{125}I]CNTF was incubated with native α_2M. The extent of binding of [^{125}I]CNTF to α_2M–methylamine was minimal. These results indicate that CNTF does not bind to either conformation of α_2M with significant affinity or that α_2M–CNTF complexes rapidly dissociate during electrophoresis.

Similar results were obtained in experiments with immobilized α_2M–methylamine. [^{125}I]CNTF (5–100 nM) was incubated with the immobilized α_2M* for 1 h at 37 °C. Binding was insufficient for analysis.

In FPLC experiments, limited binding of [^{125}I]CNTF to α_2M was observed. When 5 nM [^{125}I]CNTF was incubated with 0.7 μM native α_2M for 2 h at 37 °C, 2% of the [^{125}I]-CNTF co-eluted with native α_2M. Under equivalent conditions, 12% of the [^{125}I]CNTF was recovered with α_2M–methylamine. For comparison, similar experiments were performed with [^{125}I]NGF-β. Approximately 10% of the [^{125}I]-NGF-β was recovered with native α_2M; 70% was recovered with α_2M–methylamine. To further explore the nature of the

Table 2: BS^3 Cross-Linking Efficiencies (z) and Covalent Binding of Neurotrophins to α_2M

	α_2M–methylamine		native α_2M	
neurotrophin	z^a	(AC*/(AC* + AC)) × 100^b	z^a	(AC*/(AC* + AC)) × 100^b
NGF-β^c	0.33 ± 0.04	1.8–2.7	0.15 ± 0.06	1.1–1.5
BDNF	0.34 ± 0.04	3.5–5.6	0.24 ± 0.06	0.7–1.9
NT-3	0.48 ± 0.04	1.3–3.2	0.30 ± 0.02	0.9–1.9
NT-4	0.14 ± 0.12	1.4–3.4	0.12 ± 0.02	0.8–1.7

^{a} Mean ± SEM, n = 4. ^{b} Range of values from four experiments (nine α_2M concentrations per experiment). ^{c} Data from Crookston *et al.* (1994).

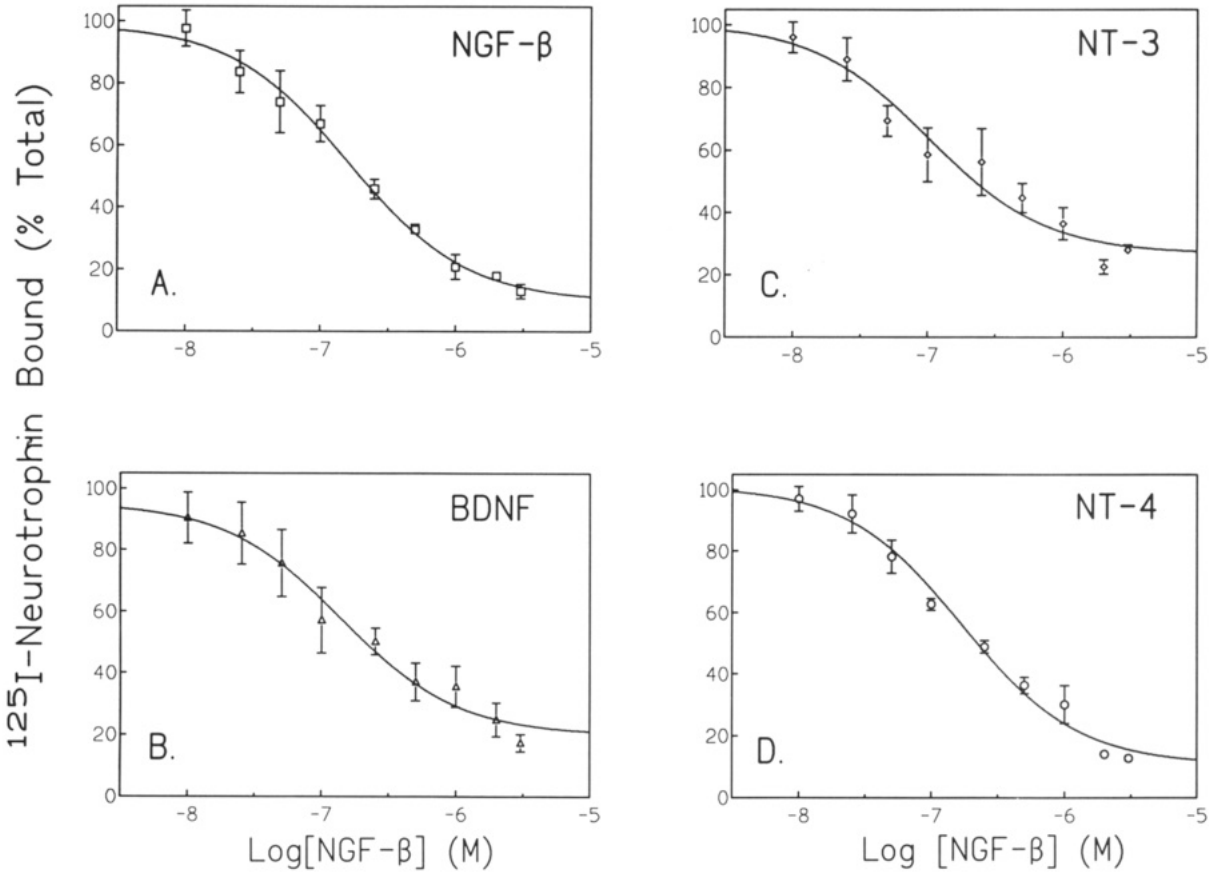

FIGURE 4: Competitive binding of unlabeled NGF-β and [^{125}I]neurotrophins to immobilized α_2M–methylamine. [^{125}I]NGF-β (43 nM, panel A), [^{125}I]BDNF (18 nM, panel B), [^{125}I]NT-3 (23 nM, panel C), and [^{125}I]NT-4 (9 nM, panel D) were incubated in wells with immobilized α_2M–methylamine. Unlabeled NGF-β (0.1–3.0 μM) was included in the incubation buffer. Binding studies were conducted in PBS containing 1 mg/mL BSA for 1 h at 37 °C. The presented results are from three independent experiments with duplicate determinations in each. Error bars represent 1 SEM.

[^{125}I]CNTF–α_2M–methylamine complex, material which was recovered from the FPLC column was subjected to SDS–PAGE. Between 25 and 45% of the CNTF–α_2M–methylamine complex was covalent, as suggested by the stability of the complex in SDS (without reductant). Covalent binding of CNTF to α_2M–methylamine was completely reversed by the addition of reductant.

Taken together, the various experiments performed to analyze the CNTF/α_2M interaction suggest that binding is minimal and of low affinity. When CNTF–α_2M complexes form, they are probably rapidly dissociable, unless stabilized by thiol–disulfide exchange.

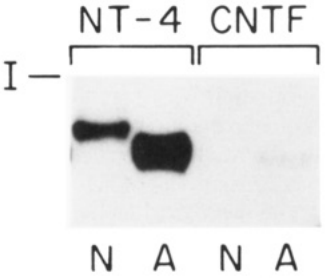

FIGURE 5: Binding of [^{125}I]NT-4 and [^{125}I]CNTF to α_2M as demonstrated by native PAGE and autoradiography. [^{125}I]NT-4 (10 nM) or [^{125}I]CNTF (20 nM) was incubated with 0.7 μM native α_2M (N) or 0.7 μM α_2M–methylamine (A) for 1 h at 37 °C and then subjected to nondenaturing PAGE. Equal amounts of radioactivity were loaded in each lane. The autoradiograph is shown. "I" demarcates the interface between the stacking and separating gels.

DISCUSSION

The present investigation expands on previous work identifying α_2M as a growth factor carrier. Binding of BDNF and NT-4 to α_2M has not been studied previously. Liebl and Koo (1993a) demonstrated that α_2M binds NT-3 and NGF-β; however, the affinities of these interactions under equilibrium conditions were not determined. Crookston *et al.* (1994) presented apparent K_D values for the binding of NGF-β to native α_2M (330 nM) and α_2M–methylamine (110 nM). By comparison with NGF-β, NT-4 binds native α_2M and activated α_2M (α_2M*) with greater affinity. Of the 11 cytokines studied to date, only transforming growth factor-β2 (TGF-β2) binds α_2M (and α_2M*) with higher affinity than NT-4 (Crookston *et al.*, 1994). The apparent K_Ds for α_2M binding to NGF-β, NT-3, and BDNF were equivalent. All four neurotrophins competed for equivalent or overlapping binding sites which are preferentially expressed in the activated α_2M structure.

The affinity of NGF-β for α_2M–methylamine was determined by two separate methods, yielding equivalent results. The major limitation in the BS^3 cross-linking method involves the assumption of one growth factor binding site per α_2M. Due to the symmetry in α_2M, we speculated that there might be two or four equivalent growth factor binding sites. If these sites are noninteracting (no cooperativity), then the apparent K_D values should be determined by plotting $[C]_e/[AC]_e$ against the reciprocal of the concentration of growth factor binding sites (instead of $1/[\alpha_2M^*]$). By contrast, the K_I values determined with immobilized α_2M–methylamine are not affected by the number of growth factor binding sites per

α_2M. The major limitations of this system include changes in the structure of α_2M–methylamine due to immobilization and accessibility of growth factor binding sites. Due to the accessibility problem, the B_{max} determined for immobilized α_2M–methylamine (1 NGF-β-binding site per α_2M*) might not be representative of α_2M* in solution. However, the equivalence of the apparent K_D (by the BS^3 method) and the K_I (by the immobilization method) suggests that there is in fact one NGF-β-binding site (with an affinity of 110 nM) per α_2M. Less likely explanations for the equivalence of the apparent K_D and K_I include the following: (1) immobilization increased the affinity of α_2M–methylamine for NGF-β; (2) radioiodination of NGF-β in the BS^3 experiments decreased the affinity of binding to α_2M–methylamine to an extent which was exactly compensated for by multiple (two or more) binding sites.

Although noncovalent neurotrophin binding to α_2M reached apparent equilibrium relatively rapidly, conversion of noncovalent complexes (AC) into covalent complexes (AC*) was slow (nearly undetectable). Covalent stabilization of AC (k_2) typically results from thiol–disulfide exchange and therefore involves primarily α_2M* since native α_2M does not have free cysteine residues (Borth, 1992). Our inability to detect significant thiol–disulfide exchange in experiments with the neurotrophins is consistent with recent observations regarding conserved structural characteristics of the neurotrophin family. Each neurotrophin is a homodimer containing six cysteine residues per monomer. In NGF-β (and presumably in the other neurotrophins) all of the cysteine residues form intrasubunit cystine bridges that are buried within the core of the structure (minimal solvent accessibility) (McDonald *et al.*, 1991). Therefore, thiol–disulfide exchange within the AC complex is unlikely to occur.

α_2M–methylamine bound all of the neurotrophins with greater affinity than native α_2M. In previous studies with TGF-β1 and platelet-derived growth factor (PDGF), we demonstrated that α_2M–methylamine provides an appropriate model of cytokine binding to α_2M–proteinase complexes which form under physiologic conditions (Hall *et al.*, 1992; Crookston *et al.*, 1993). Preferential binding of neurotrophins to α_2M* may be of particular significance since the two α_2M conformations affect the biological activity of growth factors differently. Native α_2M partially protects NGF-β and other growth factors from clearance pathways and thus enhances their stability in the circulation (Crookston *et al.*, 1993; Gonias *et al.*, 1994). By contrast, α_2M* targets growth factors to cells expressing LRP (LaMarre *et al.*, 1991; Crookston *et al.*, 1993). This interaction may mediate growth factor catabolism or regulate growth factor activity (LaMarre *et al.*, 1991; Stouffer *et al.*, 1993).

While the exact molecular interactions responsible for the binding of growth factors to α_2M remain to be characterized, a number of studies suggest a role for electrostatic interactions or ionic pairing (Gonias *et al.*, 1984; Liebl & Koo, 1993b). Such interactions are probably important only when combined with other types of protein–protein bonds since charged residues at protein surfaces show only a weak tendency to associate in H_2O, due to the high dielectric constant of this medium (Tanford, 1961). Binding of neurotrophins to $p75^{NGFR}$ (an acidic glycoprotein) also involves charged amino acids. In directed mutagenesis studies of NGF-β, lysine 32, lysine 34, and lysine 95 were important for $p75^{NGFR}$ binding (Ibanez *et al.*, 1992). These amino acids are surface-exposed; lysine 32 and lysine 34 are part of one β-hairpin loop, while lysine 95 is located in an adjacent hairpin loop, as determined from the crystal structure of NGF-β (McDonald *et al.*, 1991). The three lysine residues are conserved or replaced conservatively in the other neurotrophins (Ibanez *et al.*, 1992). Although TGF-β2 and PDGF show low sequence homology with the neurotrophins, these growth factors also include clustered basic amino acids in exposed β-turns (Daopin *et al.*, 1992; Schlunegger & Grutter, 1992; Oefner *et al.*, 1992). These regions are candidate binding sites for α_2M which are under further investigation.

In contrast with the neurotrophins, CNTF bound to α_2M weakly at best. Noncovalent association of CNTF with α_2M* was insufficient for determining a K_D by either method used in this investigation. The small amount of CNTF–α_2M–methylamine which was recovered by chromatography included a large percentage of disulfide-stabilized complex. Selective recovery of covalent CNTF–α_2M* probably reflects rapid dissociation of noncovalent complex. In addition, the thiol–disulfide exchange reaction with CNTF may be favored since this growth factor has a free cysteine residue (Manthorpe *et al.*, 1993). On the basis of the studies presented here, we propose that α_2M (even at high concentration) will not bind CNTF in the presence of CNTF-specific receptors or any other physiologically significant CNTF-binding proteins *in vivo*.

According to the neurotrophic hypothesis, developing neurons compete for limited amounts of neurotrophic factors derived from target tissues (Barde, 1989). Neurons which are unable to obtain adequate quantities of appropriate neurotrophic factors perish. Similarly, neuronal survival and axonal regrowth during regeneration require that the injured neuron acquire neurotrophic factors from its microenvironment (Heumann *et al.*, 1987; Funakoshi *et al.*, 1993). The availability of neurotrophic factors is thus a key determinant of neuronal survival during development and following injury. Recent studies have suggested that regulation of neurotrophin availability to neuronal receptors may be complex. Soluble forms of $p75^{NGFR}$ (DiStefano & Johnson, 1988; DiStefano *et al.*, 1993) and truncated, noncatalytic forms of Trk receptors (Klein *et al.*, 1990; Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993) may regulate the distribution and availability of neurotrophins. Since α_2M and the α_2M receptor (LRP) are expressed in the nervous system (Dziegielewska *et al.*, 1986; Kodelja *et al.*, 1986; Garton *et al.*, 1991; Van Gool *et al.*, 1993; Wolf *et al.*, 1992), α_2M may also influence the availability, assortment, and activity of neurotrophins.

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