Changes in the Binding of "Fast"-Form α_2-Macroglobulin to 3T3-L1 Cells after Differentiation to Adipocytes†

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ABSTRACT: Human α_2-macroglobulin (α_2M)–CH_3NH_2 specifically binds to 3T3-L1 fibroblasts and adipocytes with an apparent K_d of 0.3 nM at 4 °C. Binding to fibroblasts follows first-order kinetics only for the first 20–30 min of reaction, k_1 = 160 μM^{-1} h^{-1}, and then proceeds in a non-first-order reaction that takes 28 h to reach steady state. Receptor activity is 120 fmol of α_2M–CH_3NH_2/mg of cell protein or 60 000 molecules/cell. Binding is nondissociable. In contrast, binding to adipocytes follows first-order kinetics, k_1 = 720 μM^{-1} h^{-1}, and reaches steady state in 6–8 h. Receptor activity is 35 fmol of α_2M–CH_3NH_2/mg of cell protein or 60 000 molecules/cell. Binding is reversible with a k_2 of 0.4 h^{-1}. Control studies with 3T3-C2 cells, which do not differentiate after hormone treatment, indicate that these differences are not due to hormone treatment alone. Binding to both fibroblasts and adipocytes is specific for "fast"-form α_2M but not for native α_2M. Inhibition studies with neoglycoproteins demonstrate that binding does not occur via any of the known carbohydrate receptors. Some cross-reactivity with antithrombin III–trypsin complexes is demonstrated. Both fibroblasts and adipocytes take up and degrade α_2M–CH_3NH_2 at 37 °C. For both cell types, the concentration of α_2M–CH_3NH_2 needed for half-maximal uptake is 65 nM. Adipocytes take up more ligand and degrade less on a per cell basis than fibroblasts, a result that is consistent with inhibition of intracellular proteolysis by insulin; treatment of fibroblasts with insulin for several hours results in decreased degradation and increased uptake of ligand.

α_2-Macroglobulin (α_2M)[1] is a plasma glycoprotein of M_r 718 000 that inhibits a wide variety of proteases. Reaction of α_2M with protease results in cleavage and conformational change in α_2M and in binding and steric inhibition of the protease. This conformational change results in altered electrophoretic mobility, so-called "slow" to "fast" conversion. Conformational change without cleavage of α_2M results from reaction with primary amines such as methylamine (Starkey & Barrett, 1977; Barrett, 1981). The conformational change that occurs when α_2M reacts with methylamine or proteases is quite similar as determined by circular dichroic spectroscopy and sedimentation studies (Gonias et al., 1982).

α_2M is rapidly and specifically removed from the circulation once it has reacted with methylamine or protease (Ohlsson, 1971; Blatrix et al., 1973; Imber & Pizzo, 1981). The clearance of α_2M is mediated by a specific receptor system that has been studied in vitro in a variety of model systems including human fibroblasts (Van Leuven et al., 1978, 1979), rabbit alveolar macrophages (Kaplan & Nielson, 1979a,b), NRK fibroblasts (Dickson et al., 1981), mouse peritoneal macrophages (Imber & Pizzo, 1981; Imber et al., 1982), and rat hepatocytes and adipocytes (Glieman et al., 1983). Studies with these cells show that α_2M–protease complexes bind to the receptor with the same affinity and activity as α_2M–CH_3NH_2 complexes while "slow" form (native α_2M) does not bind (Imber & Pizzo, 1981; Kaplan et al., 1981). More, recent studies demonstrate that the K_d for the binding of α_2M–CH_3NH_2 to mouse peritoneal macrophages is 0.4 nM while α_2M–protease complexes bind with K_d values between 0.4 and 0.7 nM (Feldman et al., 1983). In addition, at 37 °C "fast"-form α_2M is endocytosed and degraded by a mechanism similar to that which degrades low-density lipoprotein (LDL) (Maxfield et al., 1978; Willingham & Pastan, 1980; Tyko & Maxfield, 1982; Wehland et al., 1982; Via et al., 1982). Like the LDL receptor, α_2M receptors are recycled (Kaplan, 1980).

The 3T3-L1 cell line has been selected for its ability to differentiate into adipocytes upon hormonal stimulation (Green & Kehinde, 1974, 1975, 1976; Green & Meuth, 1974). Initially, the cells resemble fibroblasts, but following treatment with dexamethasone, isobutylmethylxanthine, and insulin (DMI), their morphology changes to resemble that of adipocytes. In addition, primary enzymes of fatty acid synthesis (MacKall & Lane, 1977; MacKall et al., 1976; Freytag & Utter, 1980), supporting enzymes of fatty acid synthesis (MacKall & Lane, 1977; Freytag & Utter, 1980), and enzymes of glycerolipid metabolism (Kuri-Haruch & Green, 1977; Coleman & Bell, 1980; Coleman et al., 1978) are greatly increased after differentiation. Studies of two receptor systems show marked change after differentiation. Insulin receptors are increased in number on differentiated cells (Rubin et al., 1977, 1978; Reed et al., 1977). In addition, insulin itself affects the number of insulin receptors on the adipocytes, causing a decrease in receptor number (Reed et al., 1977; Ronnett et al., 1982, 1983). β-Adrenergic receptors also change after differentiation from predominantly β1 to β2. Experiments with 3T3-C2 fibroblasts, a nondifferentiating line of 3T3 cells, indicate that this change is caused by incubation with hormone rather than by differentiation per se (Lai et al., 1982).

The α_2M–receptor mediates the uptake and degradation of α_2M–protease complexes. This receptor system has also been implicated in regulation of protease secretion (Johnson et al., 1982) and superoxide production (Hoffman et al., 1983) by macrophages. In addition, Saloman et al. (1982) have shown that α_2M is a growth factor for cells in culture. Because of

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[1] Abbreviations: TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; BSA, bovine serum albumin; SBTI, soybean trypsin inhibitor; EBSS, Earl's balanced salt solution; DMI, 0.25 μM dexamethasone–0.5 mM 3-isobutyl-1-methylxanthine–10 μg/mL insulin; Man-BSA, α-D-mannosyl$_{50\ mol}$-BSA; Fuc-BSA, β-L-fucosyl$_{50\ mol}$-BSA; GlcNAc-BSA, *N*-acetyl-β-D-glucosaminyl$_{47\ mol}$-BSA; α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; β-ME, β-mercaptoethanol; TCA, trichloroacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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the demonstrated changes in insulin and β-receptor number and activity with differentiation of fibroblasts to adipocytes and because of the various physiologic functions of α_2M, we examined the changes in the α_2M–receptor interaction that occurred following cellular differentiation.

Experimental Procedures

Materials. HEPES–HCl, Sepharose 6B, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), bovine serum albumin (BSA), soybean trypsin inhibitor (SBTI), methylamine (CH_3NH_2), 2-amino-2-methyl-1,3-propanediol (ammediol), trypan blue, bovine insulin, and dexamethasone were obtained from Sigma. Earle's balanced salt solution (EBSS) (10× concentration) without bicarbonate and phenol red and either with or without calcium and magnesium was obtained from Gibco Laboratories. Also from Gibco Laboratories were Dulbecco's modified Eagle's medium and fetal bovine serum. Trypsin was obtained from Worthington, 3-isobutyl-1-methylxanthine from Aldrich, and Sepharose-coupled lactoperoxidase from P-L Biochemicals. $Na^{125}I$, carrier free, in 0.1 N NaOH, was obtained from New England Nuclear and diluted 1:10 with 0.15 M phosphate buffer, pH 7.0, prior to use. Outdated, fresh frozen plasma was obtained from the Durham Veterans Administration Hospital Blood Bank. Orosomucoid was the kind gift of the American Red Cross and was desialated by mild acid hydrolysis (Schmid et al., 1967). Fuc-BSA, GlcNAc-BSA, and Man-BSA were prepared as previously described (Stowell & Lee, 1980). Antithrombin III–trypsin complexes were prepared as described for antithrombin III–thrombin complexes except for the substitution of trypsin (Shifman & Pizzo, 1982). All other reagents were the highest grade commercially available.

Cell Culture. 3T3-L1 cells were obtained from the American Type Culture Collection; 3T3-C2 cells were the generous gift of Dr. Howard Green. Cells were grown to confluence in Nunc 24-well multidishes or, for some experiments, in Falcon 100-mm plastic dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM glutamine (standard medium). At confluence, some cells were placed in standard medium, which also contained 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin (DMI). After 48 h, this medium was replaced with standard medium containing 10 μg/mL insulin but without dexamethasone or 3-isobutyl-1-methylxanthine (Coleman & Bell, 1980). Six days after the change to standard medium with insulin, 80% of the 3T3-L1 cells showed adipocyte morphology while the 3T3-C2 cells retained their fibroblast morphology.

Preparation of Ligands. α_2M was purified from outdated fresh frozen plasma on Zn^{2+} chelate Sepharose (Kurecki et al., 1979; Imber & Pizzo, 1981). α_2M–methylamine and α_2M–trypsin complexes were prepared as previously described (Imber & Pizzo, 1981). α_2M–methylamine complexes were labeled with ^{125}I by the solid-state lactoperoxidase method (David & Reisfield, 1974). The complexes appeared as a single band on SDS–polyacrylamide gel electrophoresis (Wyckoff et al., 1977; Laemmli, 1981) under both reducing and nonreducing conditions. Autoradiography was performed on Kodak X-omat film with a Cronex Du Pont intensifying screen. Again, radiolabeled complexes appeared as a single band. During native gel electrophoresis (Nelles et al., 1980), radiolabeled complexes migrated as a single band with the mobility of the fast form of α_2M.

Binding Assay. The binding assay for the 3T3 cell monolayers was a modification of the macrophage binding assay (Imber & Pizzo, 1981). Cells were placed at 4 °C for 15–30 min and then washed 4 times with 1.0 mL of Earl's balanced salt solution with 25 mM HEPES and 10 mg/mL BSA at pH 7.3 (binding buffer). After equilibration at 4 °C for 2–4 h, the binding buffer was aspirated, and a solution of radiolabeled ligand in binding buffer was placed on the cells. After incubation with ligand, the binding reaction was terminated by aspiration of the ligand solution and three washes with 1.0 mL of binding buffer followed by three washes with 1.0 mL of Earl's balanced salt solution with 25 mM HEPES at pH 7.3.

Cells were harvested in 0.4 mL of 0.1 N NaOH and 1% SDS. The solubilized cell pellet was counted in a Scientific Products AW 14120 γ counter. Protein content was determined by the method of Lowry as modified by Peterson (Lowry et al., 1951; Peterson, 1977). In some experiments, cells were harvested by incubation with 0.5 mL of 0.5 mg/mL trypsin in 5 mM EDTA at 37 °C for 15 min. Reaction was terminated by addition of 0.5 mL of 0.5 mg/mL soybean trypsin inhibitor. Cells were counted for γ radioactivity, and cell number was determined by microscopic examination with a hemocytometer.

Nonspecific binding determined by incubation of ligand with 5 mM EDTA in the binding buffer was the same as that determined by inclusion of a 100-fold excess of unlabeled ligand. However, since incubation of cells with EDTA caused loss of 3T3 cells from the monolayer, nonspecific binding was determined by incubation of radiolabeled ligand in the presence of a 100-fold molar excess of unlabeled ligand. In all cases, nonspecific binding was less than 20% of the total binding.

We considered the possibility that α_2M from the fetal calf serum might be bound to the cell surface receptors. Therefore, prior to some experiments, the cells were incubated in serum-less media for 2 h at 37 °C so that any bound α_2M could dissociate or be internalized and degraded. The rate and extent of α_2M binding following such incubations was the same as that occurring in the absence of such incubations (data not shown).

Autoradiography. 3T3-L1 adipocytes in 100-mm plates were incubated with ligand at 4 °C for varying periods of time. Cells were fixed with glutaraldehyde. The monolayer was covered with photographic emulsion (Kodak), incubated in the dark for 3 weeks, and developed according to the manufacturer's instructions. The cells were then stained with Oil Red O and counterstained with hemotoxylin. Grains were counted at ×1000 magnification under oil.

On Time. 3T3 cell monolayers were incubated with ^{125}I-α_2M–CH_3NH_2 at 4 °C for various times. Specific binding was plotted as a function of time. On times were determined according to the equation $V_0 = k_1[L]R_t$, where V_0 is the initial rate of binding, [L] is the free ligand concentration, and R_t is the total receptor activity determined in binding isotherms. Alternatively, k_1 was calculated according to the equation

$$\frac{\ln ([L]/R)}{[L]_0 - R_t} = k_1 t + \frac{\ln ([L]_0/R_t)}{[L]_0 - R_t}$$

where [L] is free ligand concentration, R is unbound receptor activity, R_t is receptor activity, and $[L]_0$ is free ligand concentration at time = 0. A plot of

$$\frac{\ln ([L]/R)}{[L]_0 - R_t}$$

vs. time yields a straight line of slope k_1 (Rodbard, 1973). When both methods were used with the same data, values agreed to within 25% of each other.

Off Time. 3T3 cell monolayers were incubated with saturating concentrations of ^{125}I-α_2M–CH_3NH_2. After preincu-

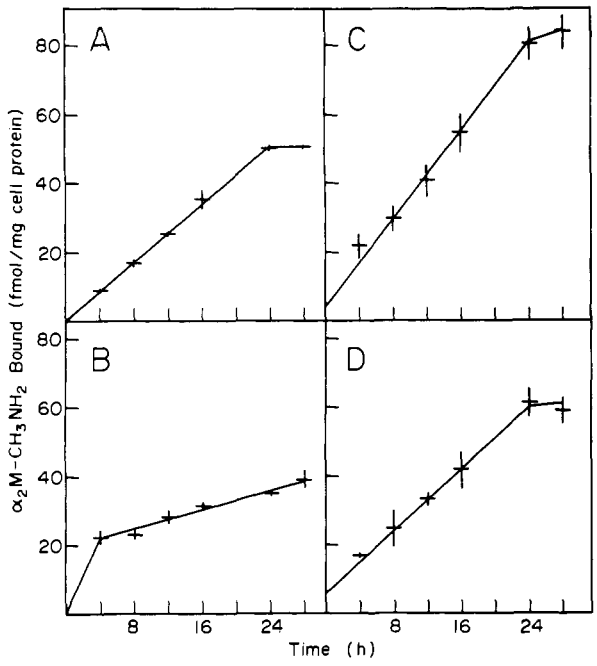

FIGURE 1: Time course of binding of ^{125}I-α_2M–CH_3NH_2 to 3T3-L1 cells. Cell monolayers were incubated with 0.5 nM ^{125}I-α_2M–CH_3NH_2 at 4 °C for varying times. Specific binding was measured as a function of time for (A) 3T3-L1 fibroblasts, (B) 3T3-L1 adipocytes, (C) 3T3-C2 fibroblasts before DMI treatment, and (D) 3T3-C2 fibroblasts after DMI treatment. The data shown are the mean and experimental variation of triplicate points. Nonspecific binding, determined by inclusion of 100-fold excess unlabeled α_2M-CH_3NH_2 in the incubations, was less than 10% of total binding for all points.

bation of varying time, unbound ligand was removed. Cell pellets were washed and then incubated with an excess of unlabeled ligand. At various times, the cell pellets were harvested and counted for γ radioactivity. The plot of ln $[R[L]/(R[L]_0)]$ vs. time yields a straight line of slope k_2, where $R[L]$ is the ligand remaining bound after a given time and $R[L]_0$ is the initial ligand bound (Rodbard, 1973).

Uptake Studies. 3T3-L1 fibroblast or adipocyte monolayers were washed 4 times with 1.0 mL of filter-sterilized binding buffer at 37 °C. The cells were allowed to equilibrate at 37 °C for 1–2 h. Binding buffer was aspirated and replaced with 0.3–0.5 mL of a solution of radiolabeled ligand in binding buffer. After incubation for various times, the solution of radiolabeled ligand was aspirated, cooled to 4 °C, and made 10% in trichloroacetic acid (TCA). The solution was left at 4 °C for 10–20 min and then centrifuged at 4 °C in a Beckman microfuge for 5 min. The supernatant was aspirated and counted for γ radioactivity. After aspiration of radiolabeled ligand solution, the cell monolayers were immediately washed 3 times with 1.0 mL of the binding buffer at 4 °C and then washed 3 times with 1.0 mL of EBSS with 0.025 M HEPES, pH 7.3, 4 °C. Cell monolayers were harvested, counted for γ radioactivity, and quantified as described for binding studies.

Results

Time Course of Binding. ^{125}I-α_2M–CH_3NH_2 was incubated with cells at 4 °C for varying times. Figure 1 shows the specific binding of 0.5 nM ^{125}I-α_2M–CH_3NH_2 as a function of time. For all cells, binding reaches a steady state after 24–48 h of binding and remains constant for the next 12–24 h. These results were also obtained for ligand concentrations of 5 and 10 nM. Undifferentiated 3T3 L1 fibroblasts show a linear increase of binding as a function of time over the entire 24 h. In contrast, differentiated cells showed an initial rapid binding of about 60% of the final ligand bound followed by a slow increase in binding over the next 20 h. This result was obtained for five experiments and over all the concentrations tested.

Table I: Distribution of ^{125}I-α_2M–CH_3NH_2 according to Cell Type for Two Different Binding Times[a]

time of binding (h)	cell type	no. of cells counted	grains counted	grains/cell
4	adipocyte	128	7103	55.5
4	fibroblast	24	521	21.7
16	adipocyte	111	7080	63.7
16	fibroblast	27	1596	59.1

[a] Cell monolayers were incubated at 4 °C with ^{125}I-α_2M–CH_3NH_2 for 4 or 16 h, fixed in glutaraldehyde, exposed to photographic emulsion, developed, stained with Oil Red O, and counterstained with hemotoxylin. Grains were counted under ×1000 magnification. For both times, 20% of the cells were fibroblasts and 80% were adipocytes.

We postulated that this initial rapid binding in differentiated cells was due to binding to adipocytes and that the subsequent slower binding was due to binding to the 20% of fibroblasts in the monolayer that do not differentiate followed DMI treatment. In order to test this hypothesis, ^{125}I-α_2M–CH_3NH_2 was incubated with differentiated cell monolayers for varying times, and the cells were fixed with glutaraldehyde, covered with a photographic emulsion, exposed at 4 °C for 3 weeks, developed, stained with Oil Red O, and counterstained with hemotoxylin. The number of grains associated with each cell type was determined. The results of this experiment are summarized in Table I. Twenty percent of the cells retained their fibroblast morphology after hormone treatment, a result consistent with other reports. The number of adipocyte-associated grains was essentially the same at 4 and at 16 h. However, the number of fibroblast-associated grains increased over 2-fold during this time. These results suggest that adipocytes bind α_2M–CH_3NH_2 more rapidly than fibroblasts. Further, since both the fibroblasts and adipocytes in this experiment had been treated with hormone, these results suggest that the difference in binding is due to changes in the cell during differentiation rather than a result of hormone treatment per se.

In order to further test whether this change was due to hormone treatment or to differentiation, we examined the time course of binding of ^{125}I-α_2M–CH_3NH_2 to 3T3-C2 cells before and after treatment with DMI. 3T3-C2 cells are a nondifferentiating line of 3T3 fibroblasts and so retain their fibroblast morphology even after incubation with DMI. The time course of specific binding to 3T3-C2 cells is shown in Figure 1. Like undifferentiated 3T3-L1 cells, these cells bind 20–25% of the final ligand bound in the first 4 h followed by a linear increase in binding of α_2M–CH_3NH_2 over the next 20 h of incubation. However, the time course of binding for 3T3-C2 cells is unchanged after treatment with DMI. These results are further evidence that the more rapid binding seen in 3T3-L1 cells after DMI treatment is due to changes in the cell type rather than hormone treatment per se.

Concentration Curve. The binding of α_2M–CH_3NH_2 as a function of concentration is shown in Figure 2. The data is linear on Scatchard plot (correlation coefficient >0.95) and is sigmoidal on the Klotz plot (Scatchard, 1949; Klotz, 1983). The affinity constant (K_d) of binding of α_2M–CH_3NH_2 to 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and 3T3-C2 cells both before and after DMI treatment was 0.2–0.4 nM, the same constant obtained for macrophages (Feldman et al., 1983). The receptor activity of 3T3-L1 fibroblasts was 120 fmol/mg of cell protein while that of adipocytes was 35 fmol/mg of cell protein. However, since adipocytes contain about 2.5 times

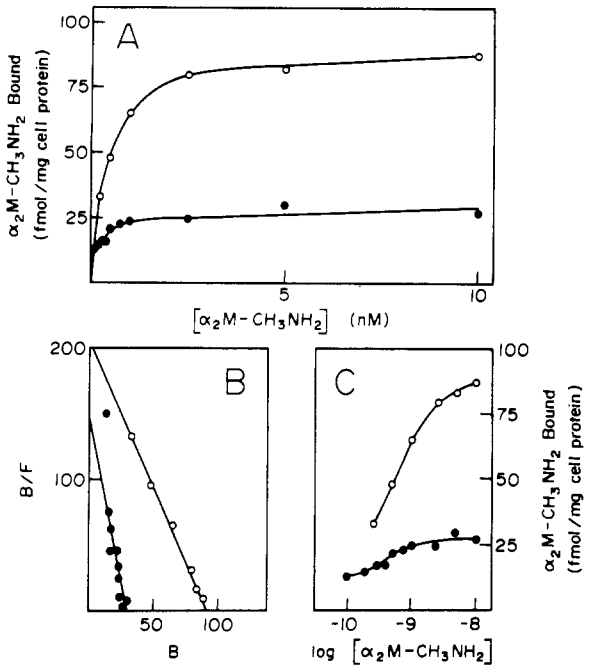

FIGURE 2: Concentration dependence of binding of ^{125}I-α_2M–CH_3NH_2 to 3T3-L1 cells. 3T3-L1 fibroblasts (○) or adipocytes (●) were incubated with varying concentrations of ^{125}I-α_2M–CH_3NH_2 for 28 h at 4 °C. The results of a typical experiment are shown. Specific binding is plotted as a function of ligand concentration (panel A) and log of ligand concentration (panel C). The same data are plotted according to the method of Scatchard in panel B. The data shown are the mean of triplicate determinations. Nonspecific binding, determined by inclusion of a 100-fold excess of unlabeled α_2M–CH_3NH_2 in the incubations, was less than 30% of total binding for all points.

Table II: Concentration Dependence of Binding of ^{125}I-α_2M–CH_3NH_2 to 3T3 Cells: Summary of Scatchard Analysis Results[a]

cell type	no. of expt	app K_d ± SD (nM)	receptor act. ± SD (fmol/mg of cell protein)
3T3-L1 fibroblasts	6	0.37 ± 0.11	123 ± 49
3T3-L1 adipocytes	3	0.18 ± 0.08	34 ± 6
3T3-C2 fibroblasts before DMI	1	0.22	170
3T3-C2 fibroblasts after DMI	1	0.21	220

[a] Varying concentrations of ^{125}I-α_2M–CH_3NH_2 were incubated with cell monolayers for 28 h at 4 °C. The data were plotted according to the method of Scatchard. The best fit line was determined by linear regression. Correlation coefficients were greater than 0.9 in all experiments summarized here.

more protein per cell than fibroblasts (Lai et al., 1981; our own observations), the receptor activity per cell is about the same for both cell types. The binding affinity (K_d = 0.2 nM) and receptor activity (200 fmol/mg of cell protein) of 3T3-C2 cells were the same before and after treatment with DMI. These results are summarized in Table II.

On Time Kinetics. Because the rate of binding changed after the differentiation of 3T3-L1 fibroblasts to adipocytes, we determined the on time constant k_1. We determined the on constant for fibroblasts from the linear portion of the time course shown in Figure 1 (4–24 h) and obtained at other concentrations of α_2M–CH_3NH_2. We assumed pseudo-first-order kinetics; that is, $V_0 = k_1 R_t[L]$. These calculations resulted in on constants that decreased as a function of increasing concentration. However, examination of the rate of binding in the first 30 min of reaction yielded a k_1 of 120 μM^{-1} h^{-1} over the concentrations tested (Table III). In contrast

Table III: Determination of On Time Constant (k_1) for 3T3 Cells: Summary of Results[a]

cell type	k_1 (μM^{-1} h^{-1}) at initial ligand concn (nM) 0.1	0.5	1	10	av
3T3-L1 fibroblasts	210	124	210	117	165
3T3-L1 adipocytes	780	790	ND	600	723
3T3-C2 fibroblasts before DMI	115	ND	120	98	111
3T3-C2 fibroblasts after DMI	100	ND	62	120	94

[a] Cell monolayers were incubated with the indicated concentrations of ^{125}I-α_2M–CH_3NH_2 for varying times. On constants were determined from the slope of the plot of specific binding vs. time (V_0), according to the equation $V_0 = k_1[L]R_t$, where [L] is the ligand concentration and R_t is the receptor activity determined in the experiments described in Figure 2 and Table II. Alternatively, on constants were calculated according to the method described by Rodbard (1973).

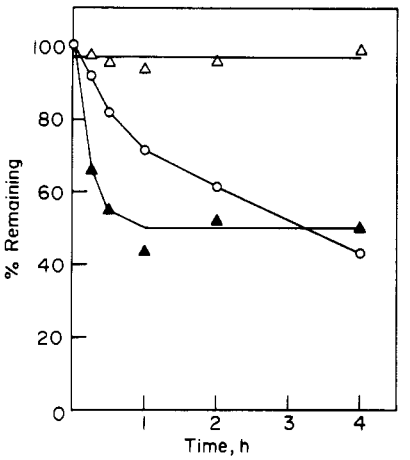

FIGURE 3: Dissociation of bound ^{125}I-α_2M–CH_3NH_2 from 3T3-L1 cells. α_2M–CH_3NH_2 was incubated with 3T3-L1 cells at 4 °C for varying times. Dissociation of bound ligand was determined by removing unbound ligand, incubating cells in an excess of unlabeled ligand for varying times, and determining cell-associated radioactivity. The percent of specifically bound ligand remaining is plotted as a function of time for 3T3-L1 fibroblasts after 28 h of initial binding (△), 3T3-L1 fibroblasts after 0.5 h of initial binding (▲), and 3T3-L1 adipocytes after 28 h of initial binding (○). The off constant, k_2, is the slope of the plot of ln $[R[L]/(R[L]_0)]$ vs. time. k_2 could not be determined for 3T3-L1 fibroblasts after initial binding of 28 h, but the k_2 for initial binding of 0.5 h is 1 h^{-1}. The k_2 for dissociation of ligand from 3T3-L1 adipocytes is 0.4 h^{-1}.

to the results for fibroblasts, only a single component was detected for the rate of binding to 3T3-L1 adipocytes, k_1 of 680 μM^{-1} h^{-1}. Once again, 3T3-C2 cells were examined as a control. On times, determined both before and after treatment with DMI, were the same and followed pseudo-first-order kinetics with a k_1 of 120 μM^{-1} h^{-1} (Table III). These results suggest α_2M–CH_3NH_2 binds to 3T3-L1 adipocytes faster than to fibroblasts, and this difference is a result of cell changes accompanying differentiation rather than an effect of hormone treatment.

Off Time. The dissociation of ^{125}I-α_2M–CH_3NH_2 from 3T3 cells was also investigated (Figure 3). Very little dissociation was observed for 3T3-L1 fibroblasts, even after a 48-h incubation with excess unlabeled ligand. These results were the same in the presence and absence of a 100-fold molar excess of unlabeled α_2M–CH_3NH_2. In contrast, about 80% of ligand dissociated from 3T3-L1 adipocytes, k_2 = 0.4 h^{-1}. In addition, dissociation of ligand could not be demonstrated from mouse peritoneal macrophages (unpublished observations) or for rabbit alveolar macrophages (Kaplan & Nielsen, 1979a). We postulated that binding was initially pseudo first order and reversible followed by an irreversible, non pseudo first order

Table IV: Specificity of Binding of ^{125}I-α_2M–CH_3NH_2 (fmol/mg of Cell Protein)[a]

inhibitor	preadipocyte	adipocyte
	27.8 ± 2.8	12.6 ± 0.1
Fuc-BSA	28.4 ± 0.6	10.4 ± 1.5
Man-BSA	28.0 ± 1.1	12.0 ± 1.0
GlcNAc-BSA	27.3 ± 0.8	12.7 ± 0.7
ASOR	27.4 ± 0.9	12.5 ± 1.3
insulin	27.4 ± 0.6	10.6 ± 1.4
antithrombin III–trypsin	15.5 ± 0.5	6.8 ± 0.2
α_2M–CH_3NH_2	1.3 ± 0.2	0.3 ± 0.1
α_2M–trypsin	1.5 ± 0.2	0.4 ± 0.1

[a] 3T3-L1 fibroblasts and adipocytes were incubated with 0.5 nM ^{125}I-α_2M–CH_3NH_2 at 4 °C for 28 h in the presence of a 400-fold molar excess of unlabeled inhibitors. Values reported for the amount of labeled ligand bound are the mean and experimental range of triplicate determinations.

reaction. Dissociation of about 40% of ligand could be demonstrated after a shorter initial incubation. These results are consistent with that interpretation (Figure 3).

We postulated that failure of the ligand to dissociate was due to formation of a covalent bond between ligand and receptor. ^{125}I-α_2M–CH_3NH_2 was incubated with 3T3-L1 fibroblast monolayer. Unbound ligand was removed and the cell pellet solubilized in gel buffer. SDS–polyacrylamide gel electrophoresis and autoradiography of the solubilized cell pellet and of ligand prior to binding are shown in Figure 4. We could not demonstrate any evidence of a covalent receptor–ligand interaction.

Specificity of Binding. The specificity of ^{125}I-α_2M–CH_3NH_3 binding to both fibroblasts and adipocytes was examined in competition studies. ^{125}I-α_2M–CH_3NH_2 (0.5 nM) was incubated with cells in the presence of unlabeled inhibitors. The results of incubations with a 400-fold molar excess of various neoglycoproteins and other ligands are summarized in Table IV. Our results confirm the earlier studies on macrophages from this laboratory (Imber & Pizzo, 1981) showing no inhibition of ^{125}I-α_2M–CH_3NH_2 binding by these neoglycoproteins. Some inhibition of binding of ^{125}I-α_2M–CH_3NH_2 occurred in incubations with antithrombin III–trypsin complexes.

We also examined inhibition of binding of ^{125}I-α_2M–CH_3NH_2 by native α_2M and α_2M–trypsin complexes. These results are shown in Figure 5. α_2M–trypsin complexes inhibited binding of labeled α_2M–CH_3NH_2 with the same dose dependence as unlabeled α_2M–CH_3NH_2. In contrast, native α_2M inhibited with 100 times less affinity than fast forms. This result is consistent with some reactivity of native α_2M with the receptor. However, since most preparations of native α_2M contain some fast form and since there is spontaneous conversion of slow to fast form over time, this inhibition is more likely due to contamination of the native preparation with a small (1%) amount of fast form (Van Leuven et al., 1978).

Uptake Studies. The time course of the uptake and degradation of ^{125}I-α_2M–CH_3NH_2 is shown in Figure 6. Uptake is linear for the first 20–30 min and reaches a plateau at 3–4 h. Generation of TCA-soluble material is linear, correlation coefficient >0.94. A summary of the steady-state level of uptake and rate of generation of TCA-soluble radioactivity is shown in Table V. On a per cell basis, both cell lines take up similar amounts of ^{125}I-α_2M–CH_3NH_2 at steady state. However, adipocytes show a decreased rate of degradation of the radiolabeled ligand.

The concentration dependence of uptake over the first 45 min is shown in Figure 7. A double-reciprocal plot of the data

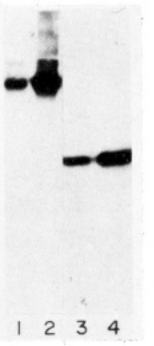

FIGURE 4: SDS–PAGE mobility of ^{125}I-α_2M–CH_3NH_2 after binding to 3T3-L1 fibroblasts. ^{125}I-α_2M–CH_3NH_2 was incubated with 3T3-L1 fibroblasts at 4 °C for 28 h. After removal of unbound ligand, the cell pellet was solubilized in SDS gel buffer and then subjected to Laemmli PAGE and autoradiography. Lanes 1 and 2 are unreduced samples; lanes 3 and 4 are reduced with βME. Lanes 2 and 4 are the samples before binding to cells; lanes 1 and 3 are the samples after binding to cells.

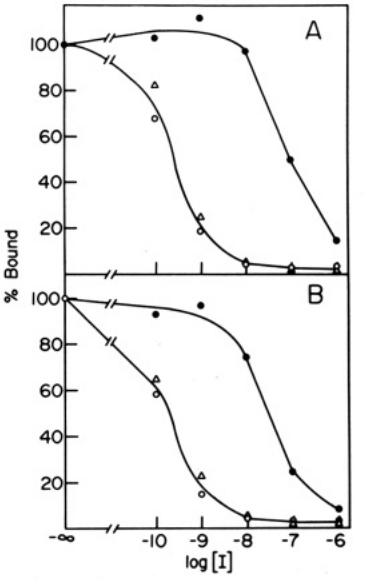

FIGURE 5: Inhibition of binding of α_2M–CH_3NH_2 to 3T3-L1 cells by native α_2M, α_2M–CH_3NH_2, and α_2M–trypsin. 3T3-L1 fibroblasts (panel A) and adipocytes (panel B) were incubated at 4 °C for 28 h with 0.1 nM ^{125}I-α_2M–CH_3NH_2 in the presence of varying concentrations of unlabeled inhibitors: native α_2M (●), α_2M–CH_3NH_2 (△), and α_2M–trypsin (○). The percent of total binding of labeled ligand in the presence of unlabeled inhibitors is plotted as a function of the log of the inhibitor concentration.

Table V: Uptake and Degradation of ^{125}I-α_2M–CH_3NH_2 by 3T3-L1 Fibroblasts and Adipocytes

cell type	[^{125}I-α_2M–CH_3NH_2] (nM)	steady-state uptake (pmol/10^6 cells)	rate of degradation [pmol (10^6 cells)$^{-1}$ min^{-1}]
fibroblasts	50	2.19 ± 0.11	1.7
	300	4.38 ± 0.62	4.1
adipocyte	50	2.66 ± 0.26	1.1
	300	5.14 ± 0.44	2.4

[a] 3T3-L1 fibroblasts and adipocytes were incubated with 50 or 300 nM ^{125}I-α_2M–CH_3NH_2 at 37 °C for various times, and cell-associated and TCA-soluble radioactivities were determined. Generation of TCA-soluble radioactivity was linear with respect to time, correlation coefficient >0.95. Uptake reached a steady state at about 3–4 h.

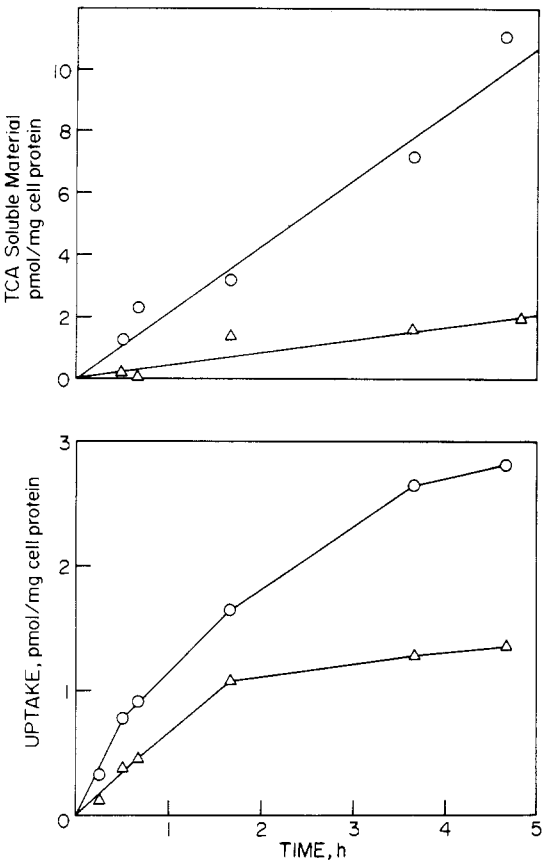

FIGURE 6: Time course of uptake of ^{125}I-α_2M-CH_3NH_2. A total of 50 nM ^{125}I-α_2M-CH_3NH_2 was incubated with 3T3-L1 fibroblasts (O) and adipocytes (Δ) at 37 °C for varying times. Reaction was terminated by removal of ligand solution and precipitation with 10% TCA. TCA-soluble material in the ligand solution is shown as a function of time in the top panel. The cell pellet was immediately washed with buffer at 4 °C and the cell-associated radioactivity determined. Cell-associated ligand is plotted as a function of time in the bottom panel.

is linear (correlation coefficients >0.95). The maximum uptake at this time is 2.1 pmol/mg of cell protein for fibroblasts and 1.3 pmol/mg of cell protein for adipocytes. The concentration at which half-maximal uptake occurs is the same for both cells, 65 nM.

We postulated that the smaller rate of degradation in the adipocytes was due to inhibition of proteolysis by the insulin used in the differentiation protocol (Draznin & Trowbridge, 1982). In order to test this hypothesis, we incubated 3T3-L1 fibroblasts in 10 μg/mL insulin for varying times. We then measured the uptake and degradation of ^{125}I-α_2M-CH_3NH_2 in 1 h in the absence of insulin. The results are shown in Table VI. The cell-associated radioactivity increased while TCA-soluble material decreased as the time of incubation with insulin increased.

Table VI: Effect of Insulin on Uptake and Degradation of α_2M-CH_3NH_2 by 3T3-L1 Fibroblasts[a]

hours of insulin pretreatment	av ± SD (pmol/mg of cell protein) cell-associated radioactivity	TCA-soluble counts
0	0.50 ± 0.04	4.6 ± 0.47
0.5	0.55 ± 0.11	4.7 ± 0.52
1	0.64 ± 0.10	4.4 ± 0.48
2	0.66 ± 0.10	4.5 ± 0.20
3	0.82 ± 0.36	3.6 ± 0.14
4	0.83 ± 0.48	3.7 ± 0.35

[a] 3T3-L1 fibroblasts were incubated with 10 mg/mL insulin at 37 °C for the indicated time. The monolayers were then incubated with 50 nM ^{125}I-α_2M-CH_3NH_2 for 1 h in the absence of insulin. Cell-associated and TCA-soluble radioactivities were determined. Values reported are the mean and standard deviation of quadruplicate points.

Discussion

These studies demonstrate a specific, saturable receptor for fast-form α_2M on 3T3-L1 fibroblasts and adipocytes. The receptors on these two cell lines differ by the rate at which fast-form α_2M binds to the receptor.

The binding of α_2M-CH_3NH_2 to 3T3-L1 fibroblasts takes 24–28 h to reach a steady state while binding to adipocytes takes about 4 h to reach steady state. The time needed to reach steady state in the 3T3-L1 fibroblasts is much longer that that reported for other cell lines; the next longest time to steady state is 8 h for NRK fibroblasts (Dickson et al., 1981). In order to further characterize the difference in binding, we determined on time rates for the two cell lines. The k_1 determined for fibroblasts was about 20% of the k_1 for adipocytes. These differences could be an indication of a change in the receptor itself or in the receptor milieu as the cells differentiate. Even though the receptor activity per cell remains the same after differentiation, the activity per milligram of cell protein decreases by about 2.5-fold. A different density of receptors could account for the different rates of interaction of α_2M-CH_3NH_2 with the receptor. In addition, changes in the cell membrane following differentiation might cause a difference in the orientation or availability of the receptor.

Evidence against a change in the receptor per se is the similarity of receptor affinity and specificity in the two cell lines. A plot of ligand bound as a function of concentration is hyperbolic on Michaelis–Menten plot, linear on Scatchard plot, and sigmoidal on Klotz plot. The concentration of α_2M that gives half-maximal binding (apparent K_d) is 0.2–0.4 nM. This value is in good agreement with K_d values reported by other workers: 0.54 nM in human fibroblasts (Van Leuven et al., 1979), 0.2 nM in rabbit alveolar macrophages (Kaplan & Nielsen, 1980), and 0.4 nM in mouse peritoneal macrophages (Feldman et al., 1983). The receptor activity of 100 fmol/mg of cell protein for fibroblasts and 40 fmol/mg of cell protein for adipocytes is about 60000 molecules of α_2M-CH_3NH_2 per cell for both lines as compared to previous reports of 11500 per cell for a human fibroblast line (Mosher & Vaheri, 1980), 110000 per cell in rabbit alveolar macrophages (Kaplan & Nielson, 1981a), 10500 per cell in NRK fibroblasts (Dickson et al., 1981), and 24000 per cell in mouse peritoneal macrophages (Imber et al., 1982).

Ligand dissociates from adipocytes with a $k_2 = 0.4\ h^{-1}$. In an ideal receptor system, $K_d = k_2/k_1$ (Rodbard, 1973). For adipocytes, $k_2/k_1 = 0.56$ nM is in good agreement with the K_d measured in the binding isotherms, 0.20 nM. Dissociation of bound ligand from fibroblasts at steady state could not be detected even after incubation of the monolayer for 48 h in the presence of excess unlabeled ligand. We detected no evidence that this failure to dissociate is due to covalent bond formation between ligand and receptor as has been described for insulin (Clark & Harrison, 1982). It also is unlikely that failure to dissociate is due to internalization of ligand. These studies were performed at 4 °C, and in most cell systems, internalization cannot occur at temperatures below 15 °C (Silverstein et al., 1977). In addition, some dissociation could be detected in the presence of 5 mM EDTA, although loss of cells from the monolayer under these conditions made this effect impossible to quantify. The results obtained for fibroblasts, failure of ligand to dissociate in the presence of

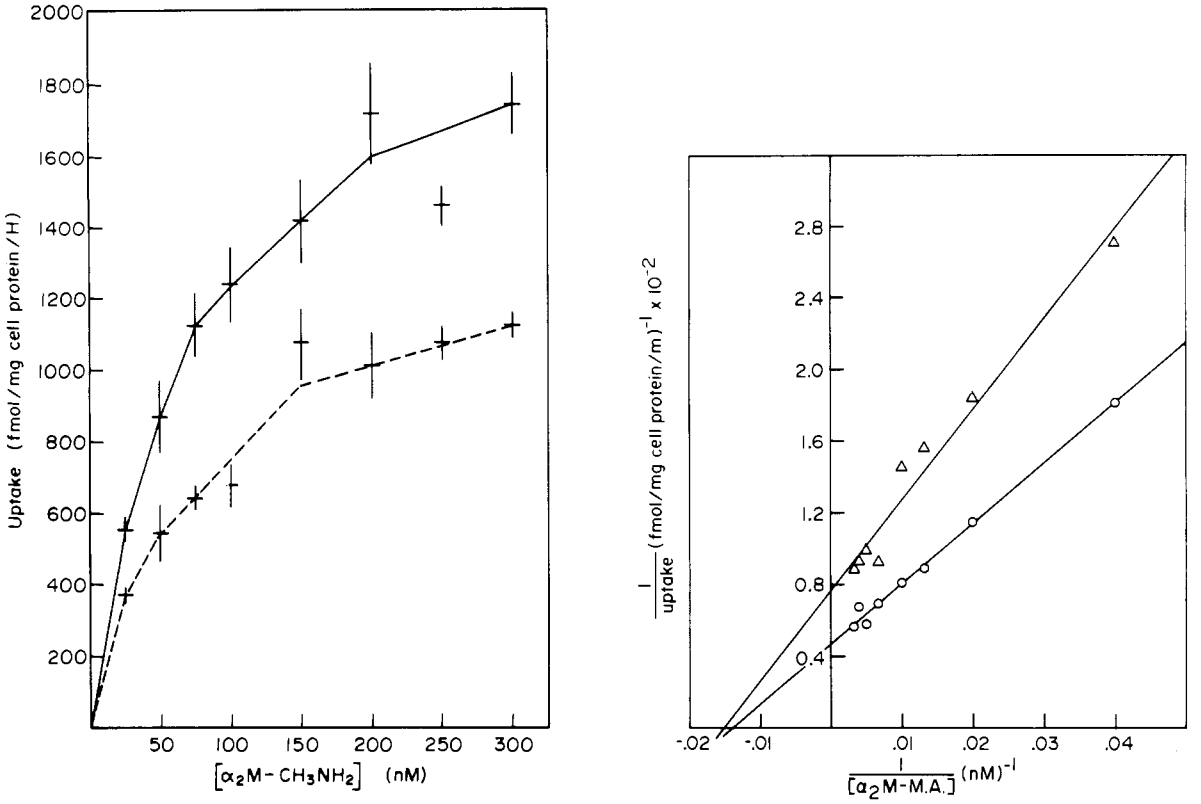

FIGURE 7: Concentration dependence of uptake of ^{125}I-α_2M–CH_3NH_2 by 3T3-L1 cells. Varying concentrations of ^{125}I-α_2M–CH_3NH_2 were incubated with 3T3-L1 fibroblasts (O) or adipocytes (Δ) at 37 °C for 1 h. Uptake was measured as a function of concentration as shown in the right panel. The left panel shows a double-reciprocal plot of concentration vs. uptake. The points shown are the mean and experimental range of triplicate determinations. The lines on the double-reciprocal plot were determined by linear regression with correlation coefficients greater than 0.95.

excess cold ligand and dissociation in the presence of EDTA, are the same as those obtained for rabbit alveolar macrophages (Kaplan & Nielson, 1979a,b).

Failure to demonstrate dissociation of the ligand suggests that the parameters obtained by the Scatchard plot are not the true dissociation constants and receptor numbers defined by Scatchard (Scatchard, 1949). In this system, a linear Scatchard plot means only that ligand bound is a hyperbolic function of free-ligand concentration. The apparent K_d refers to the concentration of ligand at which half-maximal binding occurs. The receptor affinity is the maximum amount of ligand that binds to the cells under the conditions of the experiment and is a function not only of receptor number but also receptor density and orientation and the ligand-receptor recognition site (Kaplan, 1981). Conversely, the nonlinear Scatchard plot obtained by some investigators (Dickson et al., 1981) does not prove the presence of a second, low-affinity, high-capacity receptor. Such plots must always be interpreted with care since the nonlinearity is in a portion of the curve subject to large errors (Peters & Pingoud, 1982; Munson & Rodbard, 1982). This is especially true when the system being studied involves partially or totally irreversible binding (DeMeyts, 1976).

The specificity of the receptor interaction is the same for both cell lines. Unlabeled α_2M–CH_3NH_2 and α_2M–trypsin both inhibit binding of ^{125}I-α_2M–CH_3NH_2 to the same extent at the same concentrations. Since the concentration of ^{125}I-α_2M–CH_3NH_2 in the inhibition studies is less than its K_d, the amount of unlabeled ligand decreasing binding of the labeled ligand by half is an estimate of the K_d of the competing ligand. Concentrations of α_2M–CH_3NH_2 and α_2M–trypsin causing half-inhibition of binding are about 0.4 nM. Not only do the two ligands have similar K_d values but also the K_d of unlabeled ligand is the same as that of labeled ligand. Therefore, ^{125}I-α_2M–CH_3NH_2 is a valid model for studying the binding of unlabeled fast-form α_2M.

Previous studies demonstrate that the conformations of α_2M–CH_3NH_2 and α_2M–trypsin are identical with regard to mobility in native PAGE (Starkey & Barrett, 1977), circular dichroic spectra (Gonias et al., 1982), and sedimentation coefficients (Gonias et al., 1982). However, differences in the conformations of α_2M–CH_3NH_2 and α_2M–protease have been demonstrated with the more sensitive technique of differential scanning calorimetry (Cummings et al., 1984). Data from this laboratory (Imber & Pizzo, 1981; Feldman et al., 1983) and from Kaplan's group (Kaplan et al., 1981) indicate that α_2M–protease and α_2M–CH_3NH_2 interact with the cell surface receptor with the same affinity and activity. In this paper, we have also shown that α_2M–CH_3NH_2 and α_2M–trypsin bind to the receptor with the same affinity. We conclude that the receptor recognition sites on the two fast forms are indistinguishable in this system.

Native α_2M inhibits binding by 50% at about 50 nM. This inhibition could indicate a weak interaction of α_2M with the receptor but is more likely due to a small (about 1%) contamination of native with fast form as proposed by Van Leuven et al. (1979). Neoglycoproteins and asialoorosomucoid do not inhibit binding as was shown by Imber and Pizzo in mouse peritoneal macrophages (Imber & Pizzo, 1981). There is some inhibition of binding by antithrombin III–trypsin complexes. We have also noted inhibition of antithrombin III–trypsin endocytosis by α_2M–CH_3NH_2 (Fuchs et al., 1984). The basis for this cross-reactivity is unknown.

Uptake studies with 3T3-L1 fibroblasts and adipocytes show saturation at about 200 nM, which is in good agreement with both our data with macrophages and the data of Van Leuven with fibroblasts (Van Leuven et al., 1981). The apparent K_d,

that is, the concentration at which half-maximum uptake occurs, is 65 nM as compared to 11 nM obtained by Glieman et al. (1983). Adipocytes took up slightly more ligand per cell than fibroblasts but degraded ligand at a much slower rate. We postulated that this difference might be due to inhibition of intracellular proteolysis by insulin. When fibroblasts were treated with insulin, the generation of TCA-soluble material decreased and the amount of ligand taken up increased. These changes occurred in 4 h, similar to the time reported by Draznin & Trowbridge (1982) for inhibition of intracellular proteolysis but much shorter than the time needed to cause differentiation of the fibroblasts to adipocytes. These data are consistent with the inhibition of intracellular proteolysis by insulin but do not prove that this is the basis for the difference in degradation of ligand.

Previous studies have shown that insulin receptor number increases as 3T3-L1 cells differentiate from fibroblasts to adipocytes, a change consistent with the known metabolic function of adipocytes (Rubin et al., 1977, 1978; Reed et al., 1979). We examined the changes occurring in another receptor system as 3T3-L1 cells differentiate.

Fast-form α_2M binds specifically and saturably to both 3T3-L1 fibroblasts and adipocytes. The receptor affinity and activity per cell are the same for both cell types. However, the k_1 of α_2M binding to adipocytes is 5 times that for fibroblasts. This change is probably a result of the altered receptor milieu accompanying differentiation rather than a change in the receptor per se.

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Registry No. Insulin, 9004-10-8.

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Förster-Type Energy Transfer as a Probe for Changes in Local Fluctuations of the Protein Matrix†

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ABSTRACT: Much evidence, on both theoretical and experimental sides, indicates the importance of local fluctuations (in energy levels, conformational substates, etc.) of the macromolecular matrix in the biological activity of proteins. We describe here a novel application of the Förster-type energy-transfer process capable of monitoring changes both in local fluctuations and in conformational states of macromolecules. A new energy-transfer parameter, f, is defined as an average transfer efficiency, $\langle E \rangle$, normalized by the actual average quantum efficiency of the donor fluorescence, $\langle \phi_D \rangle$. A simple oscillator model (for a one donor–one acceptor system) is presented to show the sensitivity of this parameter to changes in amplitudes of local fluctuations. The different modes of averaging (static, dynamic, and intermediate cases) occurring for a given value of the average transfer rate, $\langle k_t \rangle$, and the experimental requirements as well as limitations of the method are also discussed. The experimental tests were performed on the ribonuclease T_1–pyridoxamine 5′-phosphate conjugate (a one donor–one acceptor system) by studying the change of the f parameter with temperature, an environmental parameter expectedly perturbing local fluctuations of proteins. The parameter f increased with increasing temperature as expected on the basis of the oscillator model, suggesting that it really reflects changes of fluctuation amplitudes (significant changes in the orientation factor, κ^2, as well as in the spectral properties of the fluorophores can be excluded by anisotropy measurements and spectral investigations). Possibilities of the general applicability of the method are also discussed.

The early work of Linderstrom-Lang (Linderstrom-Lang & Schellman, 1959) suggested that protein molecules are not rigid, solidlike entities but rather show conformational fluctuations. Much subsequent research has been concerned with the investigation of protein dynamics. Recent reviews (Careri et al., 1975, 1979; Gurd & Rothgeb, 1979; McCammon & Karplus, 1980; Karplus & McCammon, 1981; Welch et al., 1982) summarize most of the relevant studies.

Naturally, the question arises as to the role of fluctuations in the functional properties of proteins. Heretofore, conventional experimental techniques have yielded little direct evidence thereupon. The well-established hydrogen–deuterium and hydrogen–tritium exchange methods have been applied successfully for some time in studying certain types of conformational motions (Hvidt & Nielsen, 1966; Woodward et al., 1982). For example, this technique was useful in exploring the connection between inhibitor binding and fluctuation of lysozyme (Nakanishi et al., 1973). Fluorescence quenching, a spectroscopic method, was first applied to protein dynamics by Lakowicz & Weber (1973), employing oxygen as quencher. This method was developed further by Eftink & Ghiron (1975), who used acrylamide as the quencher of protein tryptophan fluorescence instead of oxygen. Frauenfelder and co-workers developed numerous techniques for investigating protein fluctuations. Using laser flash photolysis methods, they detected internal protein fluctuations by following the kinetics of ligand rebinding [cf. Staerk & Chance (1969), Austin et al. (1975), and Beece et al. (1980)]. Recently introduced temperature-dependent X-ray crystallographic methods, which give the most accurate procedure for calculating the mean

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