Epidermal Growth Factor Binding to Human α_2-Macroglobulin. Implications for α_2-Macroglobulin–Growth Factor Interactions†

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ABSTRACT: We have examined the binding of ^{125}I-labeled human and mouse epidermal growth factors (EGF) to human α_2-macroglobulin (α_2M). In the presence of human neutrophil elastase, both mouse and human EGF bound to α_2M, whereas little binding was found to native α_2M. Binding was found to be predominantly covalent and mostly nonreducible by dithiothreitol. Greatly reduced binding was found when methylamine rather than proteinase was used to convert native α_2M to fast-form α_2M. Pretreatment of native α_2M with either proteinase or methylamine greatly reduced binding of EGF. Titration of human ^{125}I-EGF into native α_2M, in the presence of 2 equiv of proteinase, gave a gradual increase in EGF binding as a function of EGF concentration. Between 0.8 and 1.0 equiv of hEGF were bound per α_2M tetramer when 30 equiv of EGF were used. Reductive methylation of the α-amino group of mouse EGF eliminated most of the non-disulfide-mediated covalent binding. The pH dependence of binding of both mouse and human EGF to α_2M was examined and showed more EGF bound at pH 6 than at pH 9. The reduction in binding with increasing pH was mostly for the covalent nonreducible component. These results suggest that EGF can react with the reactive thiol ester of proteinase-activated α_2M by nucleophilic attack of the α-amino group and to a lesser extent by sulfide–disulfide exchange with the free SH of the cleaved thiol ester. The pH dependence is thought to result from competition with hydroxide for thiol ester cleavage. The low efficiency of incorporation of EGF found here, and similarly low efficiency of incorporation of insulin found by others, suggests that such covalent binding, although possible for many growth factors, is unlikely to be important *in vivo* as a means of efficiently complexing them to α_2M. This contrasts with specific noncovalent binding of growth factors to α_2M, such as appears to be the case for transforming growth factor β.

α_2-Macroglobulin (α_2M)[1] has long been known to be an inhibitor of proteinases. It inhibits them by the unusual mechanism of physical sequestration, which results from a large-scale proteinase-induced conformational change (Barrett & Starkey, 1973). Recently an additional role has been suggested for α_2M as a binding protein for growth factors, with proposed functions as a clearance vehicle, as a transporter, or to confer latency on the growth factor. Growth factors that have been shown to bind to α_2M are transforming growth factor β (O'Connor-McCourt & Wakefield, 1987; Huang et al., 1988; McCaffrey et al., 1989; Danielpour & Sporn, 1990; Philip & O'Connor-McCourt, 1991; Webb et al., 1992), platelet-derived growth factor (Huang et al., 1984), tumor necrosis factor (Wollenberg et al., 1991), interleukin 1β (Borth & Luger, 1989; Borth et al., 1990a,b), interleukin 6 (Matsuda et al., 1989), basic fibroblast growth factor (Dennis et al., 1989), nerve growth factor (Ronne et al., 1979), and insulin (Sottrup-Jensen et al., 1981; Chu et al., 1991).

Whereas the mechanism of proteinase inhibition by α_2M is accepted to involve noncovalent trapping [though non-essential covalent cross-linking also occurs frequently (Sottrup-Jensen et al., 1990)] and to be a very efficient process, with close to 100% successful inhibition, the mechanism(s) by which growth factors bind to α_2M and the efficiency of such interactions are poorly defined. To assess the possible physiological significance of α_2M–growth factor interactions it is necessary to understand the nature, specificity, and efficiency of such processes. Although many reports have documented an apparent interaction, either between purified components or upon addition of the growth factor to serum or plasma, few have provided quantitation or elucidation of the conditions necessary for binding. One example is for binding of insulin, for which it was found that binding required proteinase activation, that binding was covalent and involved the residues that constitute the thiol ester in native α_2M, and that a maximum stoichiometry of 1 insulin per α_2M subunit could be achieved in a manner that was linearly dependent on the concentration of insulin (Chu et al., 1991). However, the efficiency of incorporation was low, requiring approximately 30 equivalents of insulin to achieve maximum incorporation into complex with α_2M. The low efficiency of binding raises concerns about the specificity of such a reaction and thus of its possible importance *in vivo*. To test the possibility that many covalent growth factor–α_2M interactions are nonspecific and result from the presence of exposed nucleophilic side chains on the growth factor, we have examined the binding of a growth factor (epidermal growth factor) that had not previously been reported to bind but which could be studied in forms containing 0, 1, or 3 amino groups. We report here that human and mouse EGF bound covalently to α_2M, under conditions similar to those required for binding of other growth factors and insulin. Binding appeared to be mediated mostly by the α-amino group of EGF and to involve attack on the proteinase-

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[1] Abbreviations: TGF, transforming growth factor; PDGF, platelet-derived growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; TNF, tumor necrosis factor; α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNE, human neutrophil elastase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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activated thiol ester. Competition between EGF and proteinase for binding to the thiol ester via amino group attack was minimized by using as the proteinase human neutrophil elastase, an enzyme without lysine residues (Sinha et al., 1987). The efficiency of EGF binding was similar to that found for binding of insulin to α_2M, but this level is judged to be too low to be physiologically significant.

MATERIALS AND METHODS

Polyacrylamide Gel Electrophoresis. Samples for PAGE analysis were mixed with an equal volume of (1) nondenaturing sample buffer, (2) SDS sample buffer, or (3) SDS sample buffer containing dithiothreitol, for gels run under native, SDS nonreducing, and SDS reducing conditions, respectively. Nondenaturing 5% polyacrylamide gels were run according to the procedure of Davis (1964). SDS–polyacrylamide gels were run according to the procedure of Laemmli (1970). The α_2M samples for denaturing gels were incubated for 45 min at 37 °C in the presence of SDS prior to loading. All gels were run for 45 min at 170 V, stained with Coomassie brilliant blue for 1 min to permit visualization of the protein bands, destained for 2 min, and dried onto paper. Radioactive bands were quantitated using a Phosphor Imager (Molecular Dynamics). The dried gels were exposed to the phosphor screen for 16 h and the radioactivity associated with α_2M bands was quantitated on the basis of the total counts of all radioactive bands in the lane. The program Image Quant, supplied with the Phosphor Imager, was used in quantitation of radioactive bands.

Proteinase and Methylamine Pretreatment of α_2M. Fast form α_2M, generated by reaction with either methylamine or proteinase, was prepared immediately prior to use. For proteinase pretreatment, native α_2M in 0.2 M HEPES, 0.05 M NaCl, and 2 mM EDTA, pH 8.0, was reacted with 2.1 equiv of human neutrophil elastase for 5 min at room temperature. The reaction was stopped by addition of PMSF to a final concentration of 1 mM, followed by incubation for 5 min prior to subsequent use. For methylamine pretreatment, native α_2M in 0.2 M HEPES, 0.05 M NaCl, and 2 mM EDTA, pH 8.0 was reacted with methylamine (0.23 M) for 110 min at room temperature.

Reductive Methylation of Mouse EGF. Reductive methylation of the α-amino group of mouse EGF was accomplished by reaction with ^{14}C-formaldehyde, followed by reduction with $NaCNBH_3$, according to the procedure of Jentoft and Dearborn (1979). The reaction was carried out at pH 6.5 in 0.1 M sodium phosphate buffer, and used a 4-fold molar excess of formaldehyde over EGF and a 10-fold molar excess of $NaCNBH_3$ over formaldehyde. The reaction with formaldehyde was allowed to proceed for 2 h in the dark. Labeled EGF was separated from free formaldehyde by extensive dialysis using tubing with a nominal molecular weight cutoff of 2000. Methyl group incorporation was quantitated from the specific activity of the labeled EGF and gave a value of 1.6 mol of label/mol of EGF, compared with the expected value of 2.0 for complete and specific dimethylation of the single primary amino group. The ^{14}C-labeled EGF was divided into two, and one part was subjected to iodination by the chloramine T procedure (Hunter & Greenwood, 1962; Greenwood et al., 1963) using nonradioactive iodine.

Reaction of α_2M with EGF. Unless noted otherwise, reactions of human α_2M with either mEGF or hEGF were carried out at pH 8.0 in 0.2 M HEPES buffer containing 0.05 M NaCl and 2 mM EDTA. Reactions with HNE were allowed to proceed for 5 min before addition of 1 mM PMSF to inactivate the proteinase, followed by a further 5 min of incubation. For reaction of α_2M with proteinase or methylamine, it was possible to confirm that complete conversion from slow to fast electrophoretic forms had occurred by examination of the SDS gels following initial Coomassie staining.

Materials. Reagents were purchased as follows: phenylmethanesulfonyl fluoride from Sigma Chemical Co., St. Louis, MO; human neutrophil elastase from Athens Research & Technology Inc., Athens, Ga.; recombinant human EGF from Calbiochem; ^{125}I-labeled recombinant hEGF (specific activity 1094 Ci/mmol) from Amersham; and ^{14}C-formaldehyde (specific activity 46.7 Ci/mol) from New England Nuclear. Both native and ^{125}I-labeled mouse EGF (specific activity 743 Ci/mmol) were gifts from Dr. Graham Carpenter, Vanderbilt University. Human α_2M was purified by zinc chelate chromatography and gel chromatography, as described previously (Dangott & Cunningham, 1982). The purity and homogeneity, with respect to slow-form versus fast-form α_2M, were checked by PAGE under denaturing and nondenaturing conditions, respectively. α_2M concentrations were determined spectrophotometrically using A^{1}_{280nm} = 8.9 (Hall & Roberts, 1978).

RESULTS

Binding of Human EGF to α_2M. ^{125}I-Labeled hEGF and human α_2M were incubated together under the following conditions, at a fixed ratio of hEGF to α_2M of 30:1 (mol/mol). α_2M was incubated with hEGF in the presence or absence of human neutrophil elastase (HNE) or in the presence of methylamine and the results were compared with those from reactions in which α_2M was pretreated with either proteinase or methylamine prior to addition of hEGF, to examine the requirement of an activated thiol ester for binding. The reaction mixtures were divided into three parts and each part was run on a separate polyacrylamide gel. One gel was run under native conditions to assess total binding, one under denaturing but nonreducing conditions to assess covalent binding, and one under denaturing and reducing conditions to assess covalent binding that did not involve disulfide bonds. The dried gels were scanned using a Phosphor Imager, and the radioactivity associated with the α_2M bands was quantitated. From the stoichiometry of hEGF associated with the α_2M bands on these three gels we calculated the number of moles of hEGF noncovalently bound per mole of α_2M, the number of moles bound by disulfide bonds, and the number of moles bound covalently but not involving disulfide bonds (Table I). We found that hEGF did not bind significantly to native α_2M. Substantial binding (0.8 mol/mol) of EGF occurred when the hEGF/α_2M mixture was incubated with 2 equiv of human neutrophil elastase (HNE). The hEGF was mostly covalently associated with the α_2M but did not involve a disulfide linkage. Pretreatment of the α_2M with 2 equiv of HNE abolished almost all of the binding of hEGF. When methylamine was used to convert α_2M from slow to fast forms, very little hEGF (0.19 mol/mol total) became associated with the α_2M. Pretreatment of the α_2M with methylamine prior to addition of hEGF further reduced the level of binding to 0.10 mol/mol.

To determine the effect of hEGF concentration on the amount of hEGF bound to α_2M, a titration was performed in which different concentrations of ^{125}I-hEGF were incubated with a fixed concentration of α_2M in the presence of 2 equiv of human neutrophil elastase. Quantitation of bound hEGF was carried out as above using three polyacrylamide gels. The

Table I: Quantitation of Binding of ^{125}I-Labeled hEGF to Human α_2M

reaction[a]	mol of ^{125}I-hEGF bound/mol of α_2M[b]		
	noncovalent	covalent S–S	other covalent
native α_2M	0.02	–0.03	0.08
α_2M + HNE (2 equiv)	0.08	–0.01	0.76
α_2M + CH_3NH_2	0.05	0.04	0.10
α_2M + HNE (2 equiv) (preincubated)	–0.02	–0.03	0.13
α_2M + CH_3NH_2 (preincubated)	0.00	0.00	0.10

[a] α_2M (0.64 μM) was incubated with 30 equiv of ^{125}I-hEGF under the conditions indicated. Where preincubation is indicated, α_2M was reacted with either HNE (2 equiv) or CH_3NH_2 (0.2 M, pH 8.0) prior to reaction with hEGF under standard conditions. [b] Moles of ^{125}I-hEGF bound was calculated from three separate SDS gels run under (1) native, (2) SDS nonreducing, and (3) SDS reducing conditions. Noncovalent binding was determined as (1)–(2), covalent S–S binding as (2)–(3), and other covalent binding as (3). Values given are the average of 2–7 separate experiments. Accuracy of the method is estimated as ± 0.10 mol of hEGF/mol.

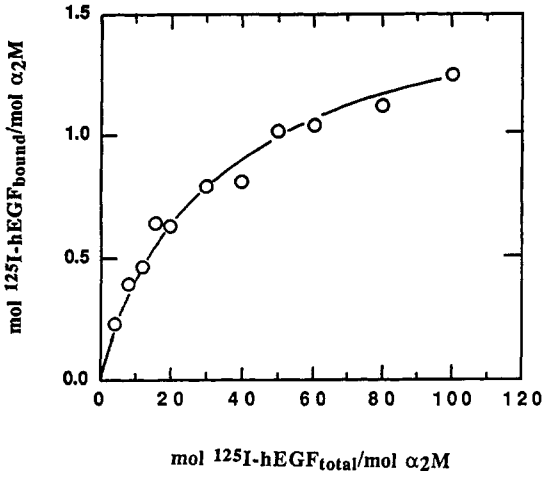

FIGURE 1: Concentration dependence of the binding of hEGF to α_2M in the presence of 2 equiv of HNE. The concentration of α_2M was kept constant at 0.64 μM. Amounts of covalent but non-disulfide-mediated binding were calculated from scanned radioactive gels as described under Materials and Methods. The data for noncovalent binding and disulfide-mediated binding are not shown.

results are shown in Figure 1 for the non-disulfide-mediated covalently bound component. The rate of increase in binding decreases as a function of concentration but does not reach saturation in the range of hEGF concentrations examined. The titration was not continued beyond 100 equiv of hEGF α_2M because of the large amounts of hEGF required and the doubtful physiological significance that binding at such high concentrations could have. Data for noncovalently bound hEGF and disulfide-linked hEGF are not shown, since the amounts bound were small (–0.05 to 0.06 mol/mol). Any significant trend was masked as a result of the relatively large errors inherent in the determination of these values as the difference of two much larger numbers.

Binding of Mouse EGF to α_2M. Since most of the hEGF bound by α_2M was found to be covalently attached but not to involve disulfide linkages, we wanted to test whether this dominant binding involved a lysyl amino group, since such linkages, involving ϵ-lysyl–γ-glutamyl bonds, have been shown to be important in covalent attachment of α_2M-entrapped proteinases (e.g., Sottrup-Jensen et al., 1990; Jacobsen & Sottrup-Jensen, 1993). Whereas hEGF has three primary amino groups (the α-amino group and two lysine ϵ-amino groups), mEGF has only one amino group, at the N-terminus.

Table II: Quantitation of Binding of ^{125}I-Labeled mEGF to Human α_2M

reaction[a]	mol of ^{125}I-mEGF bound/mol of α_2M[b]		
	noncovalent	covalent S–S	other covalent
native α_2M	–0.03	0.00	0.09
α_2M + HNE (2 equiv)	0.31	0.06	0.47
α_2M + CH_3NH_2	0.13	0.14	0.04
α_2M + HNE (2 equiv) (preincubated)	0.01	–0.01	0.16
α_2M + CH_3NH_2 (preincubated)	0.10	0.14	0.07

[a] α_2M (0.64 μM) was incubated with 30 equiv of ^{125}I-mEGF under the conditions indicated. Where preincubation is indicated, α_2M was reacted with either HNE (2 equiv) or CH_3NH_2 (0.2 M, pH 8.0) prior to reaction with hEGF under standard conditions. [b] Moles of ^{125}I-mEGF bound was calculated as described in footnote *b* of Table I.

Structurally, however, the two proteins are very similar (Cooke et al., 1987; Montelione et al., 1987). Comparison of the binding of human and mouse EGF to α_2M should therefore indicate the relative importance of α-amino and ϵ-amino groups in covalent attachment. The same conditions used for examining hEGF binding to α_2M were employed for study of the binding of mEGF to α_2M. The results are presented in Table II. A similar pattern was found for mEGF compared to hEGF. Thus, mEGF bound at significant levels (0.84 mol/mol total) in the presence of human neutrophil elastase but at much lower levels in the presence of methylamine. Pretreatment of α_2M with either proteinase or methylamine or use of native α_2M resulted in negligible binding of EGF. Much of the binding in the presence of proteinase was covalent and involved non-disulfide linkages. One difference from hEGF was the much higher fraction of the total binding in the presence of HNE that was noncovalent (0.31 mol/mol for mEGF compared to 0.01 mol/mol for hEGF). This large difference was only seen for HNE treatment, being much reduced for methylamine treatment or for pretreatment with either HNE or methylamine.

Binding of Reductively Methylated mEGF to α_2M. Reductive methylation with formaldehyde and sodium cyanoborohydride converts primary amines into tertiary amines, without greatly altering the pK_a of the amine (Jentoft & Dearborn, 1979). Since tertiary amines are not able to react with an ester or thiol ester and form an amide linkage with the carbonyl group of the ester, reductive methylation of mEGF was carried out to block the ability of the α-amino group to bind covalently to α_2M and thereby to permit determination of the contribution of this group to the observed covalent binding of mEGF to α_2M. Binding of reductively ^{14}C-methylated mEGF to α_2M was examined in the presence of HNE at a stoichiometry of mEGF to α_2M of 30:1. Quantitation of binding is shown in Table III. Reductive methylation reduced the amount of non-disulfide-mediated covalently bound mEGF binding from about 0.5 mol/mol seen with native mEGF to about 0.1 mol/mol. Such a reduction in binding would be expected if the α-amino group were involved in binding of unmethylated mEGF to α_2M.

Effect of Iodination on Covalent Binding of mEGF to α_2M. To look for any contribution to EGF binding to α_2M that was due to the iodination procedure used in modifying the EGF, comparison was made between the binding to α_2M of reductively methylated ^{14}C-labeled mEGF that had or had not been cold-iodinated by the same chloramine T procedure used for introduction of the ^{125}I label into ^{125}I-labeled EGF. This is a potential concern since artifactual binding of ^{125}I-

Table III: Binding of Reductively Methylated ^{14}C-mEGF to Human α_2M and Effect of Iodination

reaction[a]	mol of ^{14}C-mEGF bound/mol of α_2M[b]		
	non-covalent	covalent S–S	other covalent
native α_2M	–0.01	0.00	0.03
α_2M + HNE + ^{14}C-mEGF	0.18	0.16	0.12
α_2M + CH_3NH_2 + ^{14}C-mEGF	–0.06	0.09	0.03
α_2M + HNE + iodinated ^{14}C-mEGF	0.32	0.08	0.07
α_2M + HNE (preincubated) + ^{14}C-mEGF	0.00	–0.08	0.11
α_2M + CH_3NH_2 (preincubated) + ^{14}C-mEGF	0.01	0.04	0.04

[a] α_2M (0.64 μM) was incubated with 30 equiv of ^{14}C-reductively methylated mEGF under the conditions indicated. Where preincubation is indicated, α_2M was reacted with either HNE (2 equiv) or CH_3NH_2 (0.2 M, pH 8.0) prior to reaction with hEGF under standard conditions. Iodinated ^{14}C-mEGF was prepared from reductively methylated ^{14}C-mEGF by chloramine T iodination with nonradioactive iodide. [b] Moles of ^{14}C-mEGF bound was calculated as described in footnote *b* of Table I.

Table IV: pH Dependence of Binding of mEGF and hEGF to Human α_2M

EGF	pH	mol of ^{125}I-EGF bound/mol of α_2M[a]		
		noncovalent	covalent S–S	other covalent
murine	6.1	0.33	0.15	1.28
murine	6.9	0.20	0.13	0.57
murine	8.0	0.29	0.08	0.47
murine	8.8	0.25	0.06	0.26
human	6.1	0.09	0.01	1.09
human	6.9	0.09	0.00	0.71
human	8.0	0.08	–0.01	0.76
human	8.8	0.23	–0.04	0.60

[a] Moles of ^{125}I-mEGF bound was calculated as described in footnote *b* of Table I.

EGF to cell surface receptors has been reported previously (Comens et al., 1982). This was attributed to the chloramine T procedure used for introducing the ^{125}I label into the growth factor. It was proposed that the derivatization modified residues in addition to tyrosine, which could then react with side chains on the receptor. We found that the total binding of mEGF to α_2M in the presence of proteinase was unaffected by the chloramine T iodination procedure (Table III), though there appears to have been a reduction in the amount of mEGF disulfide-linked to α_2M that was compensated by an increase in noncovalent binding.

pH Dependence of EGF Binding to α_2M. The pH dependence of binding of both mEGF and hEGF to α_2M in the presence of proteinase was examined in the pH range 6–9. The same EGF α_2M ratio (30:1) was used as in the experiments described above. The amounts of noncovalent, disulfide-linked, and covalent non-disulfide-linked binding are shown in Table IV. For ^{125}I-mEGF there is an approximately 5-fold decline in non-disulfide covalent binding as the pH is increased from 6 to 9 but little change (within error) in either the disulfide-linked or noncovalently bound mEGF. For hEGF there is an approximately 2-fold decline in non-disulfide covalent binding, but again little change in the amount of noncovalent or disulfide-linked EGF except at pH 8.8, where the amount of hEGF noncovalently bound increases. At all pH values, mEGF shows considerable noncovalent association with α_2M, which is much greater than for hEGF, except at pH 8.8.

DISCUSSION

We have shown by means of polyacrylamide gels of ^{125}I-labeled EGF/α_2M reactions run under native and denaturing conditions that both mEGF and hEGF can bind to human α_2M under certain conditions. Our results support a mechanism for covalent binding that involves nucleophilic attack by the α-amino group of EGF on the carbonyl of the thiol ester. The difference in binding to native α_2M compared to α_2M in the presence of proteinase indicates the need for the α_2M to be in the activated or "nascent" state, which is found to occur for a short period subsequent to proteinase cleavage of the α_2M bait region (Sottrup-Jensen et al., 1981) and which is envisioned to involve a much more exposed thiol ester than in the native state and which is consequently more accessible to ambient nucleophiles. The small amount of disulfide-mediated binding is likely to depend on the presence of a free SH or S^- group on the α_2M molecule, which would occur only after cleavage of the thiol ester. The apparent independence of the disulfide-mediated binding of EGF from the effects of pretreatment of the α_2M with proteinase or methylamine or of reductive methylation of the EGF is consistent with this binding occurring after thiol ester cleavage and generation of a free thiol.

At first appearance, the pH dependence results (Table IV) are surprising if the mechanism of non-disulfide covalent binding of EGF to α_2M involves attack by amino groups, since the concentration of the more reactive free base form should increase as the pH is increased. However, in the presence of added proteinase or methylamine but the absence of added EGF, the thiol ester is always cleaved by the available nucleophiles H_2O, OH^-, and NH_2 (from methylamine or proteinase). Addition of EGF results in competition between these nucleophiles and nucleophiles on EGF. In the present study competition would have been only between the EGF amino groups and H_2O or OH^-, since HNE contains no lysine groups. The concentration of both OH^- and the free base form of the α-amino group of EGF are pH dependent. Since the α-amino pK_a should be close to 7 (Brown & Bradbury, 1975), the relative concentration of OH^- to free base EGF will increase as the pH is increased from about 1 pH unit below to 1 pH unit above the pK_a. This could explain the 5-fold reduction in covalent binding by mEGF as the pH is increased from 6 to 9, despite the increase in concentration of the free base form of the α-amino group, since the concentration of OH^- increases more than the concentration of the free base form of the EGF. The less pronounced pH dependence of the non-disulfide covalent binding of hEGF may reflect the presence of two additional nucleophiles in hEGF (the two lysyl ε-amino groups), which become increasingly reactive as the pH is raised toward their pK_a and the concentration of the free base form increases.

The involvement of the thiol ester groups in mediating covalent attachment of EGF imposes a maximum stoichiometry for incorporation of EGF of 4 mol per α_2M tetramer. The results of titrating hEGF into α_2M in the presence of HNE showed that, even using an optimal proteinase that contains no competing lysine groups, it was not possible to reach saturation of the four potential binding sites with 100 mol of hEGF added per α_2M tetramer. Furthermore, the binding of 0.8 mol/tetramer at a reaction ratio of 30:1 represented only 3% efficiency.

It is likely that the principal factor determining the efficiency of binding is the relative rates of the hydrolysis reaction and the nucleophilic attack by the α-amino group of the EGF. Following bait region cleavage by proteinase, the α_2M is

converted to an intermediate nascent state (Sottrup-Jensen et al., 1981). If the rate of attack by a nucleophile on the growth factor is not fast enough to out-compete solvent, the thiol ester will be hydrolyzed and there will be no further opportunity for covalent attachment by the growth factor other than by sulfide–disulfide exchange. Such a dependence on relative rates of reaction rather than on specific binding could explain why so many growth factors bind to α_2M and why stoichiometries of incorporation might vary significantly as a function of the accessibility of the α-amino group. Both mouse and human EGF have mobile N-terminal regions and exposed and reactive α-amino groups (Cooke et al., 1987; Montelione et al., 1987; Faulkner-O'Brien et al., 1991). Lysine ϵ-amino groups do not appear to play a significant role in binding EGF at physiological pH and are unlikely to do so for other growth factors for the same reason, namely, that their pK_as are too high to give a high enough concentration of the free base form at physiological pH. This contrasts with the situation with proteinases, where high degrees of covalent cross-linking occur between trapped proteinase and the glutamyl moiety of the thiol ester (Sottrup-Jensen et al., 1981, 1990). This situation is, however, fundamentally different from binding of a non-proteinase protein for which there is no specific binding site. Immediately following cleavage of the bait region and conversion of the α_2M from its native state to its much more reactive nascent state, trapped proteinases are likely to be located close to the thiol ester. This is because the proteinase must interact with the bait region, which is close to the thiol ester (Gettins et al., 1988), to cleave it. The local concentration of proteinase is therefore very much higher than the concentration of growth factor, or other protein, added at an equivalent total solution concentration. Under such circumstances a lysine ϵ-amino group on the proteinase might react at a high enough rate to out-compete solvent and give rise to an ϵ-lysyl–γ-glutamyl cross-link to the α_2M.

What are the consequences of these conclusions with regard to the physiological importance of growth factor binding and to previous reports of binding of numerous other growth factors? If we consider non-disulfide covalent binding first as a means of complex formation, it appears that only a small percentage of the growth factor can bind in the time between activation of the thiol ester through proteolysis of the bait region and cleavage of the thiol ester by competing solvent. This was found to be the case here for EGF and also for insulin binding to α_2M (Chu et al., 1990). Even though, in both cases, a significant amount of growth factor or hormone could be bound, it represented only a few percent of the total growth factor added. Disulfide-mediated binding *in vivo* must occur subsequent to bait region and thiol ester cleavages. Although the time available for such a reaction is less restricted than for reaction of an amino group with the intact nascent thiol ester, there is an upper limit imposed by the time for clearance of α_2M–proteinase complexes through receptor-mediated internalization (Feldman et al., 1983; Gliemann et al., 1983). Sulfide–disulfide exchange reactions that take many minutes to hours to give a high percentage of binding of the growth factor are thus not likely to be physiologically important. The same applies to catalysis of these reactions by nonphysiological concentrations of zinc (Borth et al., 1990a).

Non-disulfide-mediated covalent binding could be greatly increased if there were a specific noncovalent binding site for the growth factor close to the thiol ester so that the effective local concentration could be increased. Under such circumstances noncovalent binding would also be much higher than we have observed here or than was observed for insulin binding and should occur to both native and reacted α_2M. In reexamining reports of other growth factors binding to α_2M, we can use the restrictions deduced above to predict whether any of the interactions might be important *in vivo*. Insulin and EGF give very little noncovalent binding to either native or cleaved α_2M and consequently give only poor efficiency of covalent binding. PDGF and IL-1β both gave mostly covalent binding by sulfide–disulfide exchange (Huang et al., 1984; Borth et al., 1990a). However, the fraction of growth factor bound in each case was small, and in the case of IL-1β nonphysiological concentrations of zinc were required as catalyst (Borth et al., 1990a). We predict that none of these is important *in vivo*. In contrast, TGF β (Danielpour & Sporn, 1990; Philip & O'Connor-McCourt, 1991; Webb et al., 1992) and IL 6 (Matsuda et al., 1989) gave binding that has the necessary characteristics for potential physiological relevance. Binding was predominantly noncovalent, indicating a specific binding site for the growth factor, and more efficient than for the other growth factors. Since binding was noncovalent, it is less dependent on proteinase activation, and indeed binding was found to both native and fast-form α_2M.

In conclusion, we have shown that both hEGF and mEGF bound to α_2M in a predominantly covalent manner consistent with attack by the α-amino group of the growth factor on the proteinase-activated thiol ester. The efficiency of binding is similar to that found for some other growth factors but is too low for this mechanism to be important as a clearance or transport mechanism *in vivo*. The low efficiency appears to correlate with the absence of a specific noncovalent binding site for EGF. Other growth factors that appear to lack specific noncovalent binding sites for α_2M give low efficiency of predominantly covalent binding to α_2M and require proteinase activation for binding. Growth factors for which binding to α_2M may serve an important *in vivo* function appear to bind specifically and to give significant noncovalent binding to both native and proteinase-complexed α_2M. These growth factors include TGF β and IL 6.

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