Synthetic chimeras of mouse growth factor-associated glandular kallikreins.

II. Growth factor binding properties

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Abstract

Six chimeric constructs of the sequentially similar growth factor-associated kallikreins—epidermal growth factor binding protein (EGF-BP) and the γ -subunit of nerve growth factor (γ -NGF)—have been expressed, and their ability to generate complexes with epidermal growth factor (EGF) and β -NGF, analogous to the high molecular weight forms (7S NGF and HMW-EGF) found in the mouse submaxillary gland, evaluated. The chimeras are distinguished by the interchange of three regions composing the amino, middle, and carboxyl terminal regions that encompass four surface loops possibly involved in specific growth factor interactions. Native β -NGF (along with native α -NGF) formed complexes indistinguishable from naturally occurring 7S NGF, characterized by an $\alpha_2\beta\gamma_2$ structure (where β -NGF is itself a dimer), with recombinant (r) γ -NGF and with a chimera in which the amino terminal region from EGF-BP was substituted. Two other chimeras containing either the middle or carboxyl terminal regions of γ -NGF showed weaker ability to form 7S complexes. Thus, all chimeras containing two segments from γ -NGF retained at least some ability to form the 7S complex. rEGF-BP reacted weakly with EGF, but the chimera composed of the amino and middle segments of EGF-BP and the carboxyl terminal segment of γ -NGF formed a nativelike HMW-EGF complex. None of the other chimeras appeared to bind EGF. These results identify amino acid positions within each kallikrein that participate in strong growth factor interactions and demonstrate that, outside of active site contacts, different regions of the kallikreins are involved in the binding of EGF and β -NGF, respectively.

Keywords: enzyme hybrids; epidermal growth factor; high molecular weight complex formation; mutagenesis; nerve growth factor; recombinant proteins; surface loops

Epidermal growth factor binding protein and the γ -subunit of nerve growth factor are mouse glandular kallikreins with the ability to stably and reversibly associate with epidermal growth factor and nerve growth factor, respectively (Varon et al., 1967, 1968; Taylor et al., 1970, 1974).

These kallikreins have also been implicated as possible processing enzymes for the precursor forms of their specifically associated growth factors (Frey et al., 1979; Edwards et al., 1988; Blaber et al., 1989; Jongstra-Bilen et al., 1989). Thus, they may function to both generate the mature forms of these growth factors and subsequently serve as protective or carrier proteins (Nichols & Shooter, 1985).

The details of the specific interactions between the kallikreins and their associated growth factors are not known. However, several observations indicate that the primary interaction involves the carboxyl terminal arginine of the growth factor (in both cases) within the substrate binding pocket of the kallikrein (Greene et al., 1969; Server et al., 1976; Bothwell & Shooter, 1978). Other significant contacts outside of the substrate binding pocket (P_3 to P_1 positions) for both growth factor complexes have been proposed based on kinetic criteria

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Abbreviations: EGF, epidermal growth factor; EGF-BP, epidermal growth factor binding protein; α -, β -, γ -NGF, α -, β -, γ -subunits of 7S nerve growth factor (7S NGF) complex; r-EGF-BP and r- γ -NGF, recombinant forms of EGF-BP and γ -NGF; HMW-EGF complex, high molecular weight EGF complex; SDS, sodium dodecyl sulfate; NAGE, native acrylamide gel electrophoresis; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)amino]ethane sulfonic acid; BisTris, 2-bis[(2-hydroxymethyl)-amino]-2-[hydroxymethyl]-1,3-propanediol.

(Blaber et al., 1989). Because the majority of the amino acid substitutions distinguishing EGF-BP (Blaber et al., 1987) and γ -NGF (Thomas et al., 1981; Ullrich et al., 1984) are localized to specific surface loops that border on the catalytic face of this family of molecules (Bode et al., 1983), these regions have been postulated as the most likely locale for the additional binding contacts necessary for stable formation of the growth factor complexes (Blaber et al., 1989).

To identify the actual amino acid residues of the kallikreins that are required for forming the specific growth factor complexes, a series of six chimeric cDNA constructs of EGF-BP and γ -NGF were made and expressed, and the protein products were tested for their ability to form stable EGF or NGF complexes. Their kinetic properties are described in the accompanying paper (Blaber et al., 1993). In each case, the protein was subdivided into three regions, and the chimeras formed by the interchange of each segment were delineated by four generalized surface loops (Kinemages 1, 2; Blaber et al., 1990). Chimeras that would form high molecular weight complexes with either the EGF or NGF were identified, thus defining a group of amino acid residues of EGF-BP and γ -NGF that are necessary for complex formation. The results confirm that stable growth factor-kallikrein complexes, in both cases, require specific interactions outside of the substrate binding pocket, and that the location of these regions in the topology of each enzyme differs for EGF-BP and γ -NGF.

Results and discussion

NGF complex formation

The 7S NGF complex (composed of 2α , 2γ , and a β dimer) can be readily distinguished from the individual components by NAGE at pH 6.8 (Fig. 1A). By substituting the native γ -subunit with recombinant chimeras, this assay can determine which regions of the γ -subunits interact with the β polypeptide to stabilize the β - γ interface. It should be noted that these assays presuppose that no significant interactions occur between α - and γ -subunits in the 7S complex, as suggested by sedimentation velocity experiments (Silverman & Bradshaw, 1982). If it is eventually shown that significant α - γ interactions occur in the 7S complex, or that the γ -subunits have significant interactions between themselves, this could alter the interpretation of these results.

To verify the specificity of the assay and to show that 7S NGF can be formed with recombinant proteins, native EGF-BP and r- γ -NGF were substituted for native γ -NGF (Fig. 1A, lane 1; Fig. 1B, lane 4). The 7S complex was readily formed by r- γ -NGF but not with EGF-BP (r-EGF-BP was equally ineffective in forming 7S NGF; data not shown). Thus, the recombinant protein, which lacks the single carbohydrate side chain of native γ -subunit (Thomas et al., 1981) and which is incompletely processed

at internal sites (Blaber et al., 1993), is able to function appropriately in forming the 7S complex. However, its altered (slower) migration is undoubtedly due to these differences (Fig. 1A, lanes 2, 5).

Panels C-F of Figure 1 evaluate the formation of the 7S NGF complex with the six chimeras composed of the amino, middle, and carboxyl regions of EGF-BP and γ -NGF. The comparison of r- γ -NGF (Fig. 1A, lanes 1, 2), chimera BP1- γ 2- γ 3¹ (Fig. 1D, lanes 3, 4), chimera BP1-BP2- γ 3 (Fig. 2E, lanes 1, 2), and r-EGF-BP (data not shown) in fact monitors the ability of γ -NGF to form the 7S NGF complex as it is converted stepwise (from amino to carboxyl terminal) into EGF-BP. Clearly, chimera BP1- γ 2- γ 3 is able to substitute for γ -NGF in the quantitative formation of a stable 7S NGF complex, whereas BP1-BP2- γ 3 cannot. The conversion of γ -NGF into EGF-BP from the converse direction (carboxyl to amino) is represented by the series of r- γ -NGF (Fig. 1A, lanes 1, 2), chimera γ 1- γ 2-BP3 (Fig. 1C, lanes 3, 4), chimera γ 1-BP2-BP3 (Fig. 1F, lanes 1, 2), and rEGF-BP (data not shown). Chimera γ 1- γ 2-BP3 does not quantitatively form 7S NGF, although the small amount of 7S NGF observed suggests a weak interaction does occur. There is a complete lack of complex formation by chimera γ 1-BP2-BP3. However, chimera γ 1-BP2-BP3 does not resolve adequately within the electrophoretic system utilized, making it somewhat more difficult to evaluate it accurately. Nonetheless, no 7S complex was observed (for either chimera). Of the remaining two chimeras, BP1- γ 2-BP3 (Fig. 1F, lanes 3, 4) does not form a detectable complex, while chimera γ 1-BP2- γ 3 (Fig. 1F, lanes 5, 6) shows low amounts of 7S NGF, suggestive of a weak interaction.

These results, summarized in Table 1, suggest that regions 2 and 3 of γ -NGF make the maximal contributions to the formation of a stable 7S NGF complex, because chimera BP1- γ 2- γ 3 is indistinguishable from native γ -NGF in this regard. However, neither alone is able to form a complex, as judged by the absence of 7S NGF formation in experiments with the chimeras BP1- γ 2-BP3 and BP1-BP2- γ 3. Interestingly, these regions can form some complex when combined with the γ 1 domain. This observation suggests that there is some contribution provided by the amino terminal region that is obscured by the stronger interactions associated with the middle and carboxyl terminal regions. It is only evident when the γ 1 region is combined with either the γ 2 or γ 3 regions alone. The net effect of these strong and weak contributions is that when any two γ -like regions are present, there is at least some complex formation observed.

From kinetic analyses, the middle region of these two kallikreins was shown to dictate the fundamental catalytic

¹ The nomenclature for the chimeric kallikreins indicates the origin of the regions utilized in their construction, e.g., chimeric kallikrein BP1-BP2- γ 3 comprises region 1 (*Eco*R1/*Pvu*II fragment) from EGF-BP, region 2 (*Pvu*II/*Ban*II fragment) from EGF-BP, and region 3 (*Ban*II/*Pst*I fragment) from γ -NGF.

Fig. 1. NAGE assays for the determination of mouse 7S NGF complexes with native and recombinant γ -NGF, and native EGF-BP and their recombinant chimeras. The individual or combination of native or recombinant components in each lane is indicated. All gels were stained with Coomassie brilliant blue. Each panel represents a separate gel (except B and E); several lanes were excised photographically from panel F (between lanes 4 and 5) to remove redundancy. The migration positions of 7S NGF, γ -NGF, and α -NGF for each gel are marked. All components were added at 10 μ g unless otherwise indicated (value in parentheses in μ g).

nature of each molecule, with the chimeras being readily classed as BP- or γ -like based solely on the presence of region 2 (Blaber et al., 1993). Specific, but less dramatic, alterations in kinetics are contributed by regions 1 and 3. Interestingly, an analysis of the kinetic constants for the

various chimeras indicates that the ability of region 3 to form a stable NGF complex (with region 2) does not correlate with altered binding interactions within the substrate S_3 to S_1 binding sites. Thus, region 3 of γ -NGF appears to contribute binding interactions with β -NGF

Table 1. Summary of the growth factor binding characteristics for native (n) and recombinant (r) γ -NGF and EGF-BP and for the recombinant chimeras

Kallikrein	NGF complex formation ^a	EGF complex formation ^a
γ -NGF	+	—
EGF-BP	—	+
r- γ -NGF	+	—
r-EGF-BP	—	+/-
BP1-BP2- γ 3	—	+
γ 1- γ 2-BP3	+/-	—
BP1- γ 2- γ 3	+	—
γ 1-BP2-BP3	(-) ^b	(-) ^b
BP1- γ 2-BP3	(-) ^b	(-) ^b
γ 1-BP2- γ 3	+/-	—

^a + indicates quantitative formation of the growth factor complex, +/- indicates partial formation of the growth factor complex, and — indicates no detectable formation of the growth factor complex.

^b Recombinant chimera did not resolve in NAGE system at pH 6.8; complex identification is therefore tentative.

that occur outside of the S₃ to S₁ regions of the substrate binding pocket. The existence of such "external" binding interactions has been previously postulated (Blaber et al., 1989).

EGF complex formation

The complexation of EGF with the native and recombinant kallikreins, as also judged by a NAGE assay, is shown in Figure 2. This assay differs in several aspects from that used for the determination of the 7S NGF complex. Unlike the NGF complex, the HMW-EGF complex (which is composed of only two subunits: EGF and EGF-BP) does not migrate differently enough from the free kallikrein (EGF-BP) to provide a definitive assignment of complex formation based on altered mobility of the unbound kallikrein. The formation of the EGF complex is also difficult to monitor based on altered mobility of the EGF moiety because in this native gel system EGF does not migrate as a focused band but rather runs as a diffuse "smear" (Fig. 2B, lane 1). However, the formation of the HMW-EGF complex can be confirmed by the sequestration of EGF by the kallikrein using a Western blotting procedure with anti-EGF antisera. Figure 2A shows the NAGE analyses of the native, recombinant, and chimeric kallikreins with and without EGF, and Figure 2B is the Western blot of these same gels. Thus, the sequestration of EGF, as revealed by the Western blots, is only significant in the positions of kallikrein (or complex) migration shown in Figure 2A. This sequestration is, as expected, observed for naturally derived EGF-binding protein, but not for γ -NGF (Fig. 2, lanes 3, 5).

As has been reported (Blaber et al., 1990), r-EGF-BP has a lower affinity for EGF (reflected also in higher K_m

values for EGF-like substrates) than native EGF-BP. This may be due to a lack of glycosylation or altered internal processing of the recombinant kallikrein, resulting from its production in *Escherichia coli*. An effect of this lowered affinity is that formation of the HMW-EGF complex with r-EGF-BP is detectable by gel exclusion chromatography (Blaber et al., 1990) but is not as stable to NAGE (Fig. 2B, lane 7). Thus, the main band observed (Fig. 2A, lanes 6, 7) with r-EGF-BP is not complexed with EGF. However, much lesser amounts of faster material that migrate similarly to the authentic complex of native subunits (Fig. 2A, lane 3) apparently represent HMW-EGF.

Despite this limitation, analysis of the various chimeric kallikreins yielded the surprising result that one particular chimera (BP1-BP2- γ 3; Fig. 2B, lane 11) forms a stable complex detectable by NAGE. In fact, two electrophoretic forms of this chimera can be seen (Fig. 2A, lane 11) and both clearly bind EGF. However, unlike the situation with NGF, none of the other chimeras shows any affinity for EGF in this assay. Thus, it can be concluded that the binding of EGF to EGF-BP does not involve the carboxyl terminal region and that binding requires residues in both the amino and middle domains.

The enhanced ability of the recombinant chimera BP1-BP2- γ 3 to form a stable EGF complex over recombinant EGF-BP suggests that region 3 from γ -NGF increases the affinity of this chimera for EGF. Kinetic analyses have shown that chimera BP1-BP2- γ 3 is very similar to EGF-BP for a variety of EGF- and NGF-like substrates (Blaber et al., 1993). The kinetic differences between recombinant EGF-BP and chimera BP1-BP2- γ 3 are only slight, but there is a reproducible lowering of K_m values for EGF-like substrates with the chimera BP1-BP2- γ 3 (Blaber et al., 1993). Thus, the γ -subunit carboxyl terminal region appears to have improved the binding affinity within the substrate binding pocket, which apparently has compensated for the increase in K_m values associated with the nonglycosylated r-EGF-BP.

The substitution of region 1 of EGF-BP with γ -NGF has no effect upon the catalysis of a small EGF-like substrates (which probes substrate S₃ to S₁ contacts; Blaber et al., 1993). Thus, region 1 of EGF-BP, which is required for complex formation, also interacts with EGF outside of the S₃ to S₁ positions. As such, this binding site is similar to that provided, at least in part, by the carboxyl terminal domain in the binding of β -NGF. Table 1 also summarizes the EGF binding characteristics for the various chimeras.

Proteolysis of EGF

Analysis of the Western blot in Figure 2 suggests that the lengthy incubation of EGF with many of the native and recombinant kallikreins results in the generalized loss of signal for EGF and the concomitant increase in the low molecular weight bands appearing near the dye front (see

Fig. 2. NAGE assays (**A**) and corresponding anti-EGF Western blot analyses (**B**) for the determination of mouse HMW-EGF complexes with native and recombinant EGF-BP, γ -NGF, and their recombinant chimeras. Lane 1 represents EGF alone; lanes 2-9 are mixtures of native and r-EGF-BP and γ -NGF, with and without EGF; and lanes 10-21 show the six chimeras, also with and without EGF. Each panel represents three gels (lanes 1-5, lanes 6-13, and lanes 14-21). Gels in A were silver stained. The Western blots (B) of the same gels were obtained after blotting on nitrocellulose followed by incubation with rabbit anti-EGF (see Materials and methods). One microgram of EGF and 10 μ g of kallikrein were used in all cases. The arrows indicate the location of an EGF/kallikrein complex that was observed for EGF-BP and the mutant BP1-BP2- γ 3.

especially Fig. 2B, lanes 15, 17, 19, 21). One possible explanation is proteolysis of EGF. The primary sequence of EGF (Savage et al., 1972) contains three internal arginine residues (Fig. 3). These arginines are located toward the carboxyl terminus of the mature protein and are the most likely sites for proteolytic degradation of EGF by these enzymes. The residues on the amino terminal side of two of these arginines are either leucine or threonine. Threonine-arginine and leucine-arginine are in fact the carboxyl terminal residues of β -NGF and EGF, respectively. Thus,

mature EGF contains internal sequences predicted to be efficiently processed by EGF-BP and γ -NGF, respectively (Blaber et al., 1989). It is unclear from previous studies whether or not such truncated derivatives would still bind EGF-BP. It is known that modification of the carboxyl terminus of native EGF, e.g., by treatment with carboxypeptidase B to remove the arginine, prevents formation of the EGF complex with EGF-BP (Server et al., 1976). If juxtaposition of this residue to the "external" binding site is critical for HMW-EGF formation, then proteoly-

Similar potential EGF fragments are also observed in the incubations of EGF with native EGF-BP and γ -NGF (Fig. 2B, lanes 3, 5). They are more prominent with γ -NGF (where no high molecular complex forms) than with EGF-BP. Nonetheless, this suggests that degradation can proceed with even the native ligand and probably represents a side reaction that occurs, to a greater or lesser degree, with all of the kallikreins. The extent to which this contributes to loss of free EGF ligand (available to form complex) in any of the derivatives studied is unclear.

Figure 4 and Kinemages 1 and 2 show the amino acid differences between EGF-BP and γ -NGF divided into the natural segments with the three regions utilized in constructing the chimeras emphasized. Figure 5A and B di-

Region 1 of EGF-BP, which is at least partly necessary for complex formation with EGF, contains four heterogeneous amino acid positions when compared to γ -NGF. Within this set are three residues (Asn A22, Glu A23, and Ile A25) with solvent-accessible side chains on the active site face of the molecule (Fig. 4A). These amino acid positions are candidates for residues in EGF-BP that interact directly with EGF during formation of the HMW-EGF complex.

Region 3 of γ -NGF, which appears to have an analogous role in the formation of the 7S NGF complex, contains 14 heterogeneous amino acid positions in comparison

Fig. 5. Stereo diagrams of the C α trace of EGF-BP (**A**) and γ -NGF (**B**), showing the side chain atoms for the amino acid positions that differ in the two molecules used in construction of the chimeric kallikreins. The amino terminal residues are in green, the middle segment residues are in blue, and the carboxyl terminal residues are in red. The atomic coordinates are derived from comparative molecular modeling of porcine pancreatic kallikrein (Bode et al., 1983; see also Bax et al., 1993). Positions A82–A84 are not shown due to their absence in porcine pancreatic kallikrein. The numbering scheme is from Blaber et al. (1989). The active site is marked by the tripeptide substrate characteristic of each bound growth factor (displayed in a space-filling motif). Additional modeling information is provided in the accompanying paper (Bax et al., 1993).

to EGF-BP (Fig. 4). Of these positions, five are solvent accessible and on the active site face of the molecule: Thr B52, Phe C3, His C67, Asp C74, and Met C75. The residues most likely to interact with β -NGF during formation of the 7S complex are in this subset. The individual amino acids involved in these contacts are presently being identified by site-directed mutagenesis. A more detailed model of EGF-BP and its interactions with EGF is described in the accompanying paper (Bax et al., 1993).

Materials and methods

Construction of chimeras

The preparation of the EGF-BP and γ -NGF chimeras is described in the accompanying paper (Blaber et al., 1993). Briefly, construction of the chimeras involved the homologous substitution of cDNA fragments, utilizing conserved restriction sites, representing three regions of the

kallikrein protein and resulting in six unique chimeras. These three regions contain four generalized surface loops—termed loop 1, loop 2, the kallikrein loop, and the autolysis loop (Bode et al., 1983)—that have most of the amino acid replacements characterizing the two kallikreins (Evans et al., 1987; Blaber et al., 1990a). When compared to the known three-dimensional structure of the related porcine pancreatic kallikrein, they are also located near the substrate binding pocket (Bode et al., 1983) (Fig. 1). Each chimeric cDNA was inserted into an *E. coli* expression vector (Blaber et al., 1990), and the expressed protein was subsequently purified (Blaber et al., 1993).

Evaluation of growth factor–kallikrein complex formation

Formation of the high molecular weight NGF complex (7S NGF), assessed using a BisTris NAGE system (Chram-

bach et al., 1976), involved the interaction of three subunits: α -, β -, and γ -NGF (the β -subunit being the neuronally active growth factor; Varon et al., 1968; Greene et al., 1969). Native α -NGF (10 μ g) and either 10 μ g or 5 μ g of β -NGF were combined with 10 μ g of either native or recombinant kallikrein at a concentration of 1 mg/mL in 50 mM TES, pH 6.8. These concentrations represent equimolar ratios of all the proteins, except in those cases where 10 μ g of β -NGF was added, where the molar ratio of β -NGF was twice that of the other proteins. The samples were incubated approximately 30 min at room temperature and then subjected to BisTris NAGE, followed by staining with Coomassie brilliant blue. Under the conditions utilized for the BisTris NAGE analysis, β -NGF, with a basic pI value (Angeletti & Bradshaw, 1971), migrated toward the cathode and did not enter the gel. α -NGF, which has an acidic pI (Isackson et al., 1984), migrated toward the anode with the dye front of the gel. γ -NGF, EGF-BP, r- γ -NGF, r-EGF-BP, and most of the recombinant chimeras resolved within the gel. In this system, the 7S NGF moved within the gel with a characteristic low migration distance and was detected by the appearance of a single band and the concomitant decrease in free kallikrein.

Formation of the HMW-EGF complex involves only two subunits: EGF and EGF-BP (Taylor et al., 1974). Recombinant kallikrein (10 μ g) and 1 μ g of native EGF (GIBCO) were combined at a concentration of 1 mg/mL in 25 mM Tris, pH 7.5, and incubated at 37 °C overnight. These amounts of kallikrein and EGF represent approximately a 2.5-fold molar excess of kallikrein to factor. Formation of the HMW-EGF complex is a slow process and required at least 4 h, preferably overnight, to form (Taylor et al., 1974; M. Blaber, unpubl. obs.). The same BisTris NAGE system was utilized to visualize the HMW-EGF complex; however, it had only a marginally different migration distance compared to noncomplexed kallikrein. Consequently, the resolved proteins were transferred to nitrocellulose, and a Western blot procedure (Burnette, 1981), utilizing rabbit anti-mouse EGF antisera (a generous gift from Dr. Harry Haigler, Department of Physiology, University of California, Irvine) was used to detect the presence of EGF complexed with kallikrein. The blotted proteins were detected by incubation with I^{125} -radiolabeled protein A (a gift of Dr. Dan Knauer) followed by autoradiography.

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