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Review Letter

EGF-like domains in extracellular matrix proteins: localized signals for growth and differentiation?

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Multidomain proteins of the extracellular matrix (ECM) play an important role in development and maintenance of cellular organization and in tissue repair. Several ECM proteins such as laminin, tenascin and thrombospondin contain domains with homology to epidermal growth factor (EGF) and exhibit growth promoting activity. The mitogenic activity of laminin is restricted to a fragment which consists of about 25 repeating domains with partial homology to EGF and comprises the rod-like inner regions of the three short arms of the four armed molecule. The mitogenic activity does not correlate with promotion of cell attachment and neurite outgrowth for which major functional sites have been found in other regions of the laminin molecule. It is suggested that EGF-like domains in laminin, in other ECM proteins and in the extracellular portions of some membrane proteins are signals for cellular growth and differentiation. Because they are integral parts of large molecules and often of supramolecular assemblies these domains are well suited to stimulate neighboring cells in a specific and vectorial way. This concept of localized growth or differentiation signals offers an attractive mechanism for the regulation of cellular development.

Extracellular matrix; Laminin; Epidermal growth factor; Mitogen; Differentiation

1. INTRODUCTION

Much is known about small diffusible growth factors, their binding to specific cellular receptors and the mechanisms of transmembrane signalling. The complex cascade of intracellular events which leads to growth, proliferation and differentiation is being explored in conjunction with the fascinating field of oncogenes and anti-oncogenes. Less is known about the spatial organization of growth factors in tissues. Concentration gradients are important and for some growth factors binding to large components of the extracellular matrix like proteoglycans appears to be essential [1]. In addition domains with growth factor activity which are integral parts of the extracellular matrix may stimulate target cells in a much more selective way than diffusible growth factors.

Domains with a close or distant homology with epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and vaccinia growth factor (VGF) have been found in multidomain proteins of the extracellular matrix (ECM) including laminin [2], tenascin [3] and thrombospondin [4]. The latter proteins play an important role in cellular development and tissue repair. Before reviewing their structure and the limited evidence that some of these domains express mitogenic activity, some relevant data on EGF and TGF-α and their precursor forms should be recalled.

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EGF, TGFα AND THEIR PRECURSORS

The potent growth factors EGF and TGF-α [6,7]

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are small proteins (53 and 50 residues) of homologous sequences and conformations which were recently elucidated by NMR [7–9]. The proteins consist of two distinct subdomains with only few contacts between them (fig.1). The larger one comprises residues 1 to 32 and is stabilized by two disulfide bridges. The small one (residues 33–48) is stabilized by a single disulfide bond. In the C-terminal small domain the peptide chain is folded very tightly into an S-shaped structure with two short stretches of antiparallel β-structure. This unusual conformation is only possible when a number of residues including Cys 33, Gly 39 and Cys 42 (fig.1) and the distances between them are well conserved. This explains why different EGF-like sequences even if otherwise rather different are very homologous in the C-terminal region [10]. The large domain consists of two rather open loops and a third large loop with a prominent antiparallel β-structure. Here conformational sequence requirements are relaxed and loops of rather variable size may be accommodated without changing the basic core structure. Accordingly, distances between Cys residues and other features of the sequence are less conserved in the N-terminal than in the C-terminal region [10].

The native structure of EGF is stabilized by disulfide bridges and reductive cleavage causes loss of function [5,11]. Within the loops many residues are not essential for conformational stability and can be freely altered to serve different functions. Progress has been made to define residues which are functionally important by site-directed mutagenesis [12] and by comparison of peptides [10,11] but the latter approach suffers from the large dependence of function on structural integrity.

Both EGF and TGF-α are derived by processing from much larger precursor molecules, which in addition to the domains corresponding to the small diffusible growth factors contain several other EGF-like domains and some unrelated domains of unknown function [13,14]. The sequences of all known precursors of growth factors which act via the EGF-receptor [15] contain a putative membrane spanning domain. Recently it was shown [14,14a] that the TGF-α precursor is indeed a transmembrane glycoprotein that can mediate cell-to-cell stimulation by binding to the EGF receptor. Whether only the domain with the sequence of processed TGF-α or additional domains are involved is not known. In any case the results highlight the point that domains of large proteins rather than only small diffusible growth factors can be active in cellular stimulation. Internalization of the ligand-receptor complex [15] is probably not possible for membrane bound proteins and for large ECM components but the results with TGF-α precursor indicate that it is not essential for the initial steps of cellular stimulation.

2. PROTEINS OF THE EXTRACELLULAR MATRIX AND CELLULAR DEVELOPMENT

The important role of ECM components in development and maintenance of cellular organization and in tissue repair has been increasingly recognized in the past decade. Multidomain glycoproteins of the ECM [2,16,17] are similarly important in morphogenesis as the membrane bound cell attachment molecules (CAMs) [18]. As for the CAMs, focal expression at distinct stages of organ development is observed for many ECM proteins. Well studied examples are fibronectin [19,20], tenascin (also referred to as cytotactin, hexabrachion, J1) [3,21] and laminin [2,16]. Each of the three proteins exhibits diverse functions. On the one hand they are structural components of specialized forms of the ECM but on the other hand the existence of organ specific isoforms and splicing variants indicates that these proteins also possess specific biological activities. For example laminin is the first ECM component which is detected at the two cell stage in the mouse embryo [22]. It is later found as an ubiquitous component of basement membranes and at this stage it mediates cell attachment [2,16]. A homolog of laminin (s-laminin) is found with high abundance in the synaptic cleft of the neuromuscular junction [23]. This is of significance since laminin is a potent promoter of neurite outgrowth [24].

There are numerous other cellular functions which have been ascribed to laminin and other ECM proteins [2,16,25]. This diversity is reflected by the large number of cellular receptors which have been found. The exploration of receptors specific for ECM proteins started only some years ago and is expanding at a very high rate. Members of the large family of integrin receptors [26] ac-

quire their specificity for individual ECM proteins by the variable combination of a few β- with a large number of α-subunits. Some of them recognize arginine-glycine-aspartate (RGD) motifs found in ECM proteins but there are also receptors of this and other types which are not 'RGD dependent' [27]. Integrin receptors are currently looked at as mechanical mediators between ECM and cytoskeleton and so far second messenger generation by them has not been observed. In contrast to EGF-receptor [15] or other growth factor receptors the cytosolic domains of integrins are small and do not resemble a tyrosine kinase. On the other hand, phosphorylation of an integrin in the cytosolic part of its β-domain by oncogenic EGF-receptor homologs has been demonstrated [28] suggesting a communication between integrins and growth factor receptors.

3. EGF-LIKE DOMAINS IN ECM PROTEINS AND THEIR MITOGENIC FUNCTION

Large proteins of the extracellular matrix such as laminin, tenascin and thrombospondin consist of structurally and often functionally autonomous domains which often occur as modular units in several different ECM proteins. Small Cys-rich domains of distant or close homology with EGF are frequent elements. They are present in the above mentioned examples and are also found in other proteins [10].

In most cases EGF-like sequence regions are repeated manyfold in rod-like and accessible regions of the molecules. Laminin (900 kDa) has a cruciform structure with three short and one long arm composed of rod-like and globular elements [2,29]. The sequence of the three constituent polypeptide chains: B1, B2 (about 220 kDa) and A (about 450 kDa) have been elucidated for mouse [30], and in part for human [31–33] and *Drosophila* laminin [34,35]. From sequence data and electron microscopic evidence [29] it follows that the rod-like regions in the three short arms are composed of altogether about 40 EGF-like subdomains which are arranged like beads on a string in groups of 3 to 9 repeating units (fig.2).

The EGF-like domains in laminin exhibit strong internal homology. They are well conserved (average identity 60%) between mammalian and *Drosophila* laminins whereas other regions of the molecules exhibit only a much lower degree of conservation. As compared to EGF the Cys-rich repeats in laminin are somewhat larger and contain 8 instead of 6 Cys residues (fig.1). The mode of disulfide linkage is not known but it may be tentatively assumed that the first six Cys residues are linked as in EGF [5] and in factor X [36]. The connection between the 7th and 8th Cys would then add an extra loop to the laminin repeats which is not present in EGF (fig.1). Strong sequence homology with EGF and TGF-α is restricted to a region around the 5th and 6th Cys which corresponds to the small domain in EGF. There are 9 identities and 2 conservative replacements in 14 residues if TGF-α is compared with one of the repeats in the B1 chain of laminin (figs 1 and 2). Structurally important residues are also preserved in the other EGF-like repeats in laminin. Much less homology is observed between the laminin repeats and EGF in the region of the large domain of EGF. In particular, the first two cysteines of the laminin repeats are separated by a single residue whereas in the EGF structure they are linked by a flexible loop of seven residues (fig.1). These Cys residues are however in close proximity in the 3D-structure and the loop can indeed be replaced by a single residue. These and other features render it likely that also the N-terminal segments of the laminin repeats which are not clearly homologous with EGF have an EGF-like conformation (fig.1).

Tenascin is a six-armed molecule (fig.2) and each of the arms contains an uninterrupted array of 13 EGF-like repeats with strong homology to EGF [37]. Similar to laminin these arrays constitute rod-like regions of the arms. The translation per EGF-like repeat is 2 nm in tenascin and 2.5 nm in laminin as determined by electron microscopy. These values may be compared with the distance between N- and C-termini for EGF of about 2 nm [7,8]. Another protein in which EGF-like domains form part of a rod-like region is thrombospondin (420 kDa), a large glycoprotein found in platelet granules and in the ECM of the vascular wall [38]. Three repeats with strong homology to EGF have been found in each of the three arms of this molecule (fig.2).

Interestingly promotion of cell growth is observed for all three proteins. For thrombospondin, autocrine growth supportive action on smooth muscle cells is synergistic with EGF and other

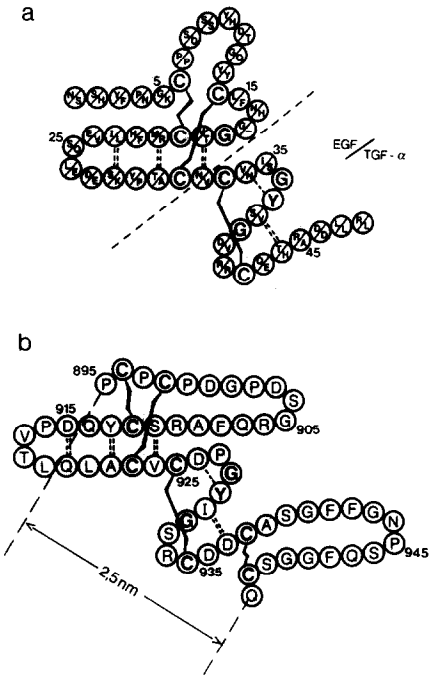

Fig.1. (a) Schematic representation of the 3D-structure of EGF and TGF-α as determined by NMR for an active derivative (residues 1–48) of human EGF [7] and mouse EGF [8]. A very similar structure is suggested for TGF-α by an NMR determination of its secondary structure [9]. (b) The hypothetical structure of one of the 40 'EGF-like' units in laminin (4th repeat in region III of the B1 chain, see fig.2). The sequences of mouse EGF (top sequence of a), rat TGF-α (bottom sequence of a, numbering for mouse EGF) [5] and of the laminin domain [30] are indicated by the one letter code. Residues which are highly conserved in EGF and TGF-α of different species are represented by bold characters and disulfide bridges by thick bars. The dashed line indicates the boundary between the large N-terminal and small C-terminal domain [7,8].

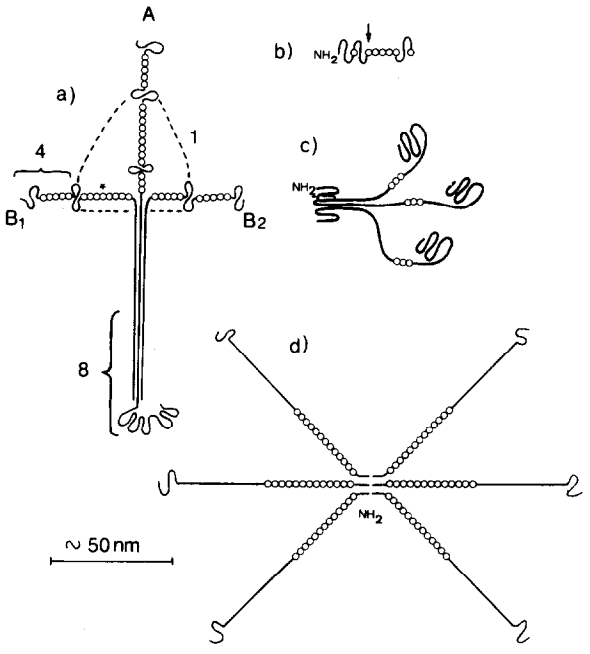

Fig.2. Schematic drawings of (a) laminin [29,30], (b) nidogen [2,42], (c) tenascin [3,21,37] and (d) thrombospondin [4,38] with EGF-like domains indicated by circles. For other details of the complex domain organization of the molecules see the above cited references. The EGF-like laminin domain whose sequence and predicted structure is shown in fig.1b is marked by an asterisk and that in nidogen which contains the RGD sequence by an arrow. Localization of fragments 1, 4 and 8 in the laminin molecules which were used in mitogenic and cell attachment studies (table 1) are indicated by numbers. Amino termini of the three chains are indicated by their designations A, B1 and B2. The three molecules are drawn approximately to scale.

growth factors [39]. Chick embryo fibroblasts and various tumor cells continue to divide in the absence of serum when grown on a tenascin substrate [40]. Promotion of growth is also reported in several studies in which cultured cells are grown on laminin substrates [25]. From such studies in which laminin (or another ECM protein) is used as a substrate and cells are grown in a medium containing fetal calf serum, indirect effects of cell attachment on growth cannot be excluded. As discussed by Kleinman et al. [25] receptors for growth factors present in the medium may become more accessible by substrate induced changes of cell shape. In a recent study [41], this problem was circumvented by the use of quiescent cells attached to plastic and by addition of laminin and fragments thereof in soluble form to a medium containing very low serum levels. Cell attachment and mitogenic activities have been measured separately for a number of fragments derived from different regions of the laminin molecule acting on a variety of cultured cells and the following results were obtained: the mitogenic response of cells to laminin and to its fragment 1 is comparable to that of EGF concerning effective concentration, magnitude, time dependence and synergetic enhancement by insulin. Laminin exhibits mitogenic activity on all cells studied except on NR6 cells which lack the EGF receptor. The activity is

localized in fragment 1 which comprises the inner short arm structures of laminin and consists of about 25 EGF-like repeats (fig.2). Fragments originating from other parts of the molecules exhibit no growth factor activity but are in part active in cell attachment (table 1). There is no correlation between mitogenic and cell attachment activity which are therefore two distinct functions of the laminin molecule presumably mediated by different receptors.

Laminin is often non-covalently associated with a 150 kDa protein referred to as nidogen, entactin or C-chain [2]. Nidogen (fig.2) also contains EGF-like domains [42] and binds tightly to the inner rod-like segments of laminin. The laminin-nidogen complex was therefore also tested for mitogenic function [41] and was found to be as active as laminin. Isolated nidogen, however, had no growth factor activity on the cells which were tested [41]. Interestingly one of its EGF-like domains contains an RGD sequence (fig.2) and it has been proposed that this sequence is recognized by an integrin receptor in the RGD dependent cell attachment on nidogen [43].

The cellular receptor involved in the growth stimulation by laminin has so far not been identified. The close resemblance of the dose response, the synergism with insulin, the activation of S6 kinase by fragment 1 and the lack of response of EGF receptor deficient NR6 cells could indicate that mitogenic mechanisms of laminin and EGF proceed in some steps via related pathways. On the other hand, laminin and its active fragments do not compete with EGF for binding to cells [41]. It is unlikely that the EGF-like regions in laminin are processed into small diffusible growth factors by cells because fragment 1 is the proteolytically least sensitive region in the molecule [2,16]. I favor the concept of a mitogenic stimulation of cells by unprocessed domains in analogy with TGF-α precursor and Notch (see below). The mitogenically active domain 1 may be accessible to cells only during early stages of tissue development before laminin is assembled into an intact basement membrane and may become exposed again after damage of basement membranes by injury. Under these conditions, laminin may express its growth factor-like function on neighbouring cells and may also act on the cells that secrete it by autocrine stimulation. Laminin domain 1 has been localized in the interior of epithelial corneal basement membrane [44] and renal tubular basement membrane [45] of adult mice and is thus very unlikely to be in direct contact with the surface of epithelial cells being attached to the basement membrane. Such cells are normally arrested in a nonproliferative phase. This indicates an advantage of growth promoting activity being present in a large structural protein allowing a localized and regulated action when compared with a diffusible small growth factor that would exert its action in a much less controlled fashion.

Table 1

Mitogenic and cell attachment activities (in parentheses) of laminin (L) and its fragments 1, 8 and 4 according to Panayotou et al. [41]

Cell line	L	1	8	4
Swiss 3T3	+ (+)	+ (+)	− (+)	−
BALB/c	+ (+)	+ (−)	− (+)	nd
PAM 212	+ (−)	+ (−)	− (−)	−
MCF-7	+	+	nd	nd
NR6	− (+)	− (+)	− (+)	nd

Swiss 3T3 and BALB/c are fibroblastoid cell lines, NR6 is a Swiss 3T3 variant cell line of different clonal origin devoid of EGF receptor, PAM 212 are mouse epidermal cells and MCF-7 human breast carcinoma cells. nd, not determined

4. ARE GENERALIZATIONS POSSIBLE?

For ECM proteins other than laminin conclusive data which would allow to attribute their mitogenic function to their domains containing EGF-like repeats are still missing. Even for laminin the function has only been localized in a relatively large fragment which in addition to its many EGF-like repeats may contain small portions of other domains (fig.2). The possibility that EGF-like domains in laminin are involved in mitogenic function may however be related to the proposed functions of repeated arrays of EGF-like units in Notch and lin-12 (reviewed in [46]). Both proteins regulate the development of certain cell lineages. A point mutation in one of the 35 EGF-like repeats of Notch has a dramatic effect on this function. As for laminin the possibility that diffusible EGF-like peptides are released by processing of the extracellular domain of Notch was discussed, but

available evidence [46] suggests that the cellular interactions are due to unprocessed extracellular domains containing the EGF-like repeats.

It is tempting to speculate that EGF-like growth factors and related domains in multidomain proteins have evolved from a common ancestor with the original function of providing signals in cellular communication. This function may have diversified into different signals for growth, differentiation, and other receptor mediated functions. The best known members of this family, EGF and TGF-α, are very important in embryonic development and this is also suggested for the stationary EGF-like domains in a number of extracellular multidomain proteins. Examples for functions apparently not related to growth or differentiation are the recognition of an EGF-like domain in urokinase-type plasminogen activator by its receptor [10] and the hypothetical role of EGF-like domains in LDL-receptor and related proteins [47]. The long arrays of EGF-like domains in many proteins were probably formed by gene duplication. A functional advantage could be the creation of multiple-binding sites, which may be important for the oligomerization of receptors or for more specific recognition. Oligomerization of the EGF receptor is considered to be essential for transmembrane signalling [48]. ECM proteins and extracellular domains of membrane bound proteins mediate between rather distant sites of ECM components and cells. The insertion of spacer elements between functional domains may therefore provide another evolutionary advantage. Many of the EGF-like domains in ECM proteins may therefore have lost their original specific function and may now only serve as structural spacer elements in rod-like regions of the molecules. This may also hold true for the many other proteins of apparently unrelated function and structure which contain EGF-like domains. It is interesting to note, however, that the list of specific receptor-mediated functions reported for EGF-like regions in these proteins is increasing [10,47]. Almost every month a new gene locus with important functions in *Drosophila* development is described which encodes for proteins with EGF-like domains. Homologs of the EGF receptor have also been found ([49] and references therein). It is therefore a reasonable working hypothesis that not all of the many EGF-like domains in ECM proteins are dull spacer units but that some of them have very interesting and specific functions.

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