Biochimica et Biophysica Acta 1824 (2012) 326–333

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Substrate kringle-mediated catalysis by the streptokinase-plasmin activator complex: Critical contribution of kringle-4 revealed by the mutagenesis approaches

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ARTICLE INFO

Article history:
Received 10 May 2011
Received in revised form 18 September 2011
Accepted 19 October 2011
Available online 25 October 2011

Keywords:
Fibrinolytic system
Pichia pastoris
Plasminogen
Plasminogen activator
Streptokinase
Catalysis

ABSTRACT

Streptokinase (SK) is a protein co-factor with a potent capability for human plasminogen (HPG) activation. Our previous studies [1] have indicated a major role of long-range protein–protein contacts between the three domains (alpha, beta, and gamma) of SK and the multi-domain HPG substrate (K1–K5CD). To further explore this phenomenon, we prepared truncated derivatives of HPG with progressive removal of kringle domains, like K5CD, K4K5CD, K3–K5CD (K3K4K5CD), K2–K5CD (K2K3K4K5CD) and K1–K5CD (K1K2K3K4K5CD). While urokinase (uPA) cleaved the scissile peptide in the isolated catalytic domain (μPG) with nearly the same rate as with full-length HPG, SK-plasmin showed only 1–2% activity, revealing mutually distinct mechanisms of HPG catalysis between the eukaryotic and prokaryotic activators. Remarkably, with SK.HPN (plasmin), the 'addition' of both kringles 4 and 5 onto the catalytic domain showed catalytic rates comparable to full length HPG, thus identifying the dependency of the "long-range" enzyme–substrate interactions onto these two CD-proximal domains. Further, chimeric variants of K5CD were generated by swapping the kringle domains of HPG with those of uPA and TPA (tissue plasminogen activator), separately. Surprisingly, although native-like catalytic turnover rates were retained when either K1, K2 or K4 of HPG was substituted at the K5 position in K5CD, these were invariably lost once substituted with the evolutionarily more distant TPA- and uPA-derived kringles. The present results unveil a novel mechanism of SK.HPN action in which augmented catalysis occurs through enzyme–substrate interactions centered on regions in substrate HPG (kringles 4 and 5) that are spatially distant from the scissile peptide bond.

1. Introduction

Clot-dissolver protein drugs, which are either the physiological plasminogen activators (pPAs), namely uPA and TPA, or the non-physiological activators (nPAs) that originate from bacteria such as *Staphylococcus* or *Streptococcus*, exert their medically beneficial influence through the activation of HPG to its proteolytically active form, HPN, which then dissolves the pathological blood clots within the circulatory system [1–3]. Unlike the pPAs which are proteases 'directly' acting on their substrate (plasminogen), the nPAs, such as SK and staphylokinase (SAK), are 'indirect' activators, in that they are protein co-factors that first combine in a 1:1 manner with plasminogen and rapidly undergo maturation to proteolytically functional activator complexes – either SAK-plasmin or SK-plasmin, respectively. These can activate HPG to HPN through specific hydrolysis of the Arg560-Val561 scissile peptide bond in substrate plasminogen, converting it to free plasmin [3]. Thus, neither SK or SAK alone, nor plasmin, which is a serine protease with essentially a trypsin-like side-chain specificity and a broad substrate preference, can activate substrate HPG to HPN by itself (which requires the highly specific proteolysis of the single scissile peptide bond in the zymogen), yet once complexed with nPAs like SK or SAK, the hitherto 'non-specific' active site of HPN gains an inordinately high order of specificity for the cleavage of the scissile peptide bond in the macromolecular substrate, HPG, which is a large, multi-domain protein [4–7]. The remarkable alteration of the macromolecular substrate specificity of HPN by the nPAs as a result of the latter's 'protein co-factor' property, has been the subject of intense investigations, and is currently thought to be due to substrate-specific loci/exosites generated on SK.HPN [6,8–13]. On the other hand, the pPAs are proteases with a high degree of intrinsic substrate specificity, and can

Abbreviations: Glu-PG, K1–K5CD (closed-conformer); Lys-PG, K1–K5CD (opened-conformer); K2–K5CD, K2K3K4K5CD; K3–K5CD, K3K4K5CD; K4K5CD, kringle 4 and kringle 5 conjugated with CD; K5CD, kringle 5 conjugated with CD; CD, catalytic domain of plasminogen; μPG, microplasminogen (catalytic domain of plasminogen); μPN, microplasmin (active form of catalytic domain of plasminogen); DEAE-Sepharose, diethylaminoethyl-Sepharose; EACA, ε-aminocaproic acid; HPG, human plasminogen; HPN, human plasmin; IBs, inclusion bodies; IPTG, isopropyl-1-thio-β-D-galactopyranoside; k_{cat}, rate of catalysis at substrate saturation; K_m, Michaelis–Menton constant; NPGB, p-nitrophenyl p-guanidinobenzoate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; SK, streptokinase; SAK, staphylokinase; SUPA, *Streptococcus uberis* plasminogen activator; TPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

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doi:10.1016/j.bbapap.2011.10.010

directly act on the macromolecular substrate to activate it with high efficiency [14–16].

Plasminogen contains five characteristic triple-loop structures called kringles, each stabilized by three disulphide bonds, linked in series to a serine protease catalytic domain of a molecular weight of around 25 kDa. The kringles are 80–90 residue modular units with very high overall 3-dimensional and amino acid sequence similarity [17,18], although these are present in a variety of functionally distinct proteins, among which are those involved in the blood coagulation and fibrinolytic pathways [19,20]. These largely participate in characteristic effector functions by providing binding sites for other plasma proteins and cellular receptors [21–25]. Although structurally homologous, the plasminogen kringle domains possess subtly different surface topologies. As a result, these exhibit different affinities as well as different specificities for the ω-amino acid ligands *e.g.* lysine and its analogs, such as EACA. The strongest binding site for EACA is provided by kringle-1 followed by kringle-4 and kringle-5 in case of human plasminogen kringles [26–30]. The kringle-3 domain of HPG does not interact with EACA to a measurable extent, whereas kringle-2 displays a very weak interaction with this ligand [31]. Primary structure alignments show that the HPG kringles share relatively high sequence similarity among themselves, varying between 50 and 58% [32].

The pathogenic bacteria secreting the protein co-factors SK and SAK are from different taxonomic lineages, namely the genera *Streptococcus* and *Staphylococcus*, respectively, these share a common attribute in that both 'prey' upon the evolutionarily distant HPG for their action *in vivo* [33,34]. Both co-factor proteins have strong affinity towards plasminogen, and form equimolar activator enzyme complexes, which then transiently bind to and activate the substrate plasminogen. Thus, activation of plasminogen by SK occurs in two distinguishable steps (i) a species-constrained step in which SK first forms a functional enzymatic complex in a species-specific manner with plasmin(ogen) [35], and (ii) a species-unconstrained step, wherein the functional activator complex can recruit a 'free' PG molecule as substrate, and activate it into plasmin irrespective of the host organism from which the PG is derived. Interestingly, even though the first step seems to provide a strong "species barrier", SK can form potentially viable complexes with trace plasmin in a non-species stringent manner that may locally activate PG and help spread the organism [35–39].

The general molecular mechanism of 'partner' plasminogen activation (which primarily involves the zymogen activation step in the initial SK-HPG complex) has been elegantly elucidated in recent years [4,6,40]. It has been shown by different groups [37,41–42] that the isolated light chain of plasminogen *i.e.* micro-plasminogen (μPG) – which is essentially the serine protease domain of full-length HPG – is capable of forming an equimolar complex with SK that shows nearly the same level of HPG activator activity as the complex between full length HPG and SK. These observations provide clear indications that kringles are dispensible for the formation of the functional 1:1 equimolar complex between SK and HPG. By contrast, it has been shown [1,42] that the extent of activation of μPG as a substrate by the SK-plasmin activator complex was much less as compared to activation of full-length HPG, indicating that kringle domains of substrate HPG are critically involved in the interaction of the macromolecular substrate with the SK.HPN enzyme. Furthermore, solution-based studies employing truncation studies on SK have unequivocally demonstrated that long-range enzyme–substrate interactions involving all the three domains of SK and the substrate kringles are important during HPG activation by the 'SK.HPN system' [1,11,43]. However, the exact nature of the participation of different kringle domains of substrate and their cognate interaction sites on the activator is not known, except for a single locus in SK (the so-called 250-loop in the beta domain), which interacts with the fifth kringle of HPG, resulting in enhanced enzyme–substrate affinity [11,43].

Recently, the contribution of kringle-5 in context of generating substrate affinity has been elegantly studied in which residues of SK-250 loop have been shown to recognize the substrate PG kringle-5 domain, exclusively for proving substrate affinity [43]. The exact contributory role of kringle-5 and other kringles of the substrate in augmenting enzyme–substrate interactions followed by catalytic potentiation, if any, remains unclear. The crystal structure of SK with only the catalytic domain of HPN, namely microplasmin (μPN) [40] bound to it, does provide a high-resolution picture of various inter-molecular interactions of the three domains of SK (α, β, and γ) with μPN during binary complex formation, but the disposition of the kringles of either partner and/or substrate HPG is entirely lacking in the structure of the SK-μPN complex. Hence, insights into the role of kringles, which appears vitally important to decipher molecular details of catalysis by SK-plasmin, must perforce be gleaned from indirect mutational and biochemical studies.

Accordingly, in the present study, we have carried out selective truncation of the kringle domains and chimeric variants of human plasminogen to explore their role in HPG activation by SK-plasmin. The results, presented below, clearly reveal the important mechanistic contributions of the kringle domains of the substrate in plasminogen activation by SK. Intriguingly, the results also point towards a phenomenon whereby the streptococci have likely 'exploited' these highly conserved structures for induction of pathogenesis.

2. Materials and methods

2.1. Design and construction of various human plasminogen derivatives and chimeric variants

The construction of the cDNA encoding the μPG and K5CD has been described elsewhere (44), for K4K5CD (sequence encompassing residues 350–791) of HPG, K3–K5CD (residues 249–791 of HPG), K2–K5CD (residues 163–791 of HPG) and K1–K5CD (residues 78–791 of HPG) see Fig. 1A and Table S1. Briefly, construction of recombinant plasmids involved the PCR amplification of the selected regions of the human cDNA clone pCMV6-XL4-HPG construct (obtained from OriGene Technologies Inc., USA), using specific upstream and downstream primers (see Table S2 under the Supporting Information section for a listing of the primers designed for the various PG derivatives) and cloning in the various expression hosts, either *E. coli* or *Pichia pastoris*. After transforming the *P. pastoris* cells with the respective HPG derivative genetic constructs, the best clones in terms of protein yields were selected using a plate-based screening methodology as described elsewhere [44] for the respective HPG derivative *viz.* K4K5CD, K3–K5CD, K2–K5CD and K1–K5CD with C-terminal hexa-histidine tags. To purify HPG derivatives from their respective culture broths, Ni-NTA affinity chromatography was carried out. The amount of proteins in the various fractions was measured using Bradford's method of protein estimation, and their purity was analyzed by SDS-PAGE. The respective pooled protein fractions were further dialyzed against the buffer containing 0.025 M sodium acetate buffer, pH 6.0, 0.1 M NaCl for 10–12 h at 4 °C to get rid of the high concentrations of imidazole as well as salt.

To check the presence of glycosylated moieties in recombinant HPG derivatives, the SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250 and in parallel, another gel was treated by glycoprotein detection kit (Sigma, USA). This detection system is essentially a modification of the well known Periodic Acid-Schiff (PAS) method, and yields magenta bands with a light pink or colorless background. The protocol for PAS staining was followed as per manufacturer's instructions. To calculate exact concentration of each recombinant plasminogen derivative, quantitative amino acid analysis was carried out (see below). For the construction of K5CD chimeric variants, splice overlap extension (SOE) methodology was used. In case of constructing chimeric variants of K5CD, first PCR was done using the human