ATP-regulated activity of the plasmin–streptokinase complex: a novel mechanism involving phosphorylation of streptokinase

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Streptokinase, an extracellular protein produced by Streptococci, is capable of activating the human fibrinolytic zymogen plasminogen. The rate of amidolytic activity of the plasminogen–streptokinase complex is greatly diminished by micromolar concentrations of ATP and heparin oligosaccharides. In addition, the plasminogen activator activity of the plasminogen–streptokinase complex is also inhibited by these effectors. ATP and heparin oligosaccharides show structural similarity, suggesting that the inhibition is caused by binding of these molecules to a common newly formed binding pocket in streptokinase, which appears after interaction with plasminogen. Ad-

dition of the bivalent cations Ca^{2+} and Mg^{2+} reverses the inhibition caused by ATP and heparin. In the presence of ATP and bivalent cations, the complex between plasminogen and streptokinase develops an autophosphorylating activity whose target is the sequence L¹⁴³TSRPAHG in the 4.5 kDa streptokinase N-terminal peptide, which is an early autolysis peptide. This streptokinase N-terminal peptide, which is essential for streptokinase activating activity, may serve, once phosphorylated, in mechanisms related to the pathogenicity of Streptococci. These studies suggest a critical role for plasminogen in regulating the activity of the streptokinase molecule.

INTRODUCTION

Streptokinase (SK), a 46 kDa extracellular protein produced by the majority of group A, C and G Streptococci, is capable of indirectly activating the zymogen plasminogen (Pg), yielding active plasmin (Pm) [1]. Although SK itself is not a proteinase, it forms a complex with Pg which then undergoes a transition by which the complex Pm–SK becomes a potent activator [2]. When SK is incubated with human Pg, one event which occurs is the specific fragmentation of SK [3]. Analysis of the SK fragments identified a fragment with a molecular mass of approximately 4.5 kDa with the N-terminal sequence IAGPE, identical to the N-terminal sequence of native SK [4], and a 36 kDa fragment of N-terminal sequence SKPFA, corresponding to residues Ser⁶⁰–Ala⁶⁴ of the native SK molecule [4]. Although the 36 kDa fragment can form a complex with human Pg at stoichiometric ratios, no significant zymogen activation is observed [4].

The Pg-activating function of SK also can be inhibited by ATP and 3',5'-AMP [5]. This unusual effect of nucleotides on proteolytic activity has been previously observed with kidney cathepsin D [6], where the activation of the proteinase by ATP may serve specifically to regulate the cleavage of parathyroid hormone [6]. However, the mechanism of ATP inhibition of Pm–SK was not investigated [5]. We now report that ATP is a potent inhibitor of Pm–SK amidolytic activity. Similarly, heparin-derived oligosaccharides which show structural similarity to ATP [7] are potent inhibitors of this activity. The inhibition in both cases is reversed by addition of bivalent cations (Mg^{2+} and Ca^{2+}). The reversal of ATP inhibition by these cations results in autophosphorylation of the peptide L⁴²TSRPAHG⁴⁹ in the SK N-terminal 4.5 kDa peptide. This event is the result of the interaction between Pg and SK, since neither Pg/Pm nor SK alone binds ATP. Pg forms a complex

with an SK fragment comprising residues Val¹⁴³–Arg²⁸⁹, but this complex is unable to develop either proteolytic or autophosphorylating activity, presumably due to the lack of the SK N-terminal region. Our experiments suggest that this novel mechanism, involving proteolysis and phosphorylation of the SK N-terminal region, may play an as yet undiscovered physiological role in the behaviour of Streptococci

EXPERIMENTAL

Materials

Adenosine 5'-[γ-thio]triphosphate (ATP[S]), ATP and other nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Val-Leu-Lys-p-nitroanilide (VLK-pNA) was purchased from Helena Laboratories (Beaumont, TX, U.S.A.). Na¹²⁵I and [γ-³²P]ATP (3000 Ci/mmol) and [¹⁴C]ATP (558 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). The synthetic octapeptide L¹⁴³TSRPAHG was purchased from Multiple Peptide Systems (San Diego, CA, U.S.A.). All other chemicals were of the highest grade available.

Proteins

Pg was purified from human plasma by affinity chromatography on L-Lys–Sephacrose [8] and separated into isoforms 1 and 2 by affinity chromatography on concanavalin A–Sephacrose [9]. Isoform 2 was used for all experiments. SK was purified from Kabikinase (Kabi Co., Stockholm, Sweden) as described by Castellino et al. [10]. The concentration of proteins was determined spectrophotometrically at 280 nm using the $A_{1\text{cm}}^{1\%}$ of

Abbreviations used: SK, streptokinase; Pg, plasminogen; Pm, plasmin; SK₁₇, the 17 kDa SK fragment comprising residues Val¹⁴³–Arg²⁸⁹; ATP[S], guanosine 5'-[thio]triphosphate; pNA, p-nitroanilide.

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16.7 for Pg [11] and 7.5 for SK [12]. Radioiodination was carried out by the method of Markwell [13]. Radioactivity was measured in an LKB 1272 γ -radiation counter. The incorporation of ^{125}I was approx. 8×10^6 c.p.m./nmol of protein.

The octapeptide LTRPAHG was coupled to ovalbumin with glutaraldehyde using the method of Kagen and Glick [14]. The octapeptide (10 mg) was dissolved in 1.5 ml of 0.4 M sodium phosphate, pH 7.5, and added to 10 mg of ovalbumin dissolved in 1 ml of water. Then, 1 ml of 20 mM glutaraldehyde was added dropwise with stirring over the course of 10 min at room temperature. The excess unreacted glutaraldehyde was blocked by adding 0.25 ml of 1 M glycine and the mixture was stirred for an additional 30 min. The excess peptide and reagent were removed by exhaustive dialysis versus sodium phosphate-buffered NaCl, pH 7.2.

The 17 kDa SK fragment (SK₁₇) comprising residues Val¹⁴³–Arg²⁸⁹ was prepared by digestion of SK with Sepharose-immobilized human Pg and then binding of the fragments generated to a (human Pg)–Sepharose column previously treated with 1 mM *p*-nitrophenyl *p*'-guanidinobenzoate as described by Rodriguez et al. [15].

Glycosaminoglycans

Heparin oligosaccharides were prepared by dissolving 10 g of hog mucosa heparin (170 units/mg) and 34.5 g of NaNO₂ in 80 ml of water at pH 6.0. The pH of this solution was adjusted to 1.5 with 6 M HCl and maintained at 1.5 by dropwise addition of 6 M HCl or 2 M Na₂CO₃ until the reaction reached completion (cessation of N₂ evolution; about 6 min). When the reaction was complete, the pH was adjusted to 8.5 with 2 M Na₂CO₃, and the mixture was centrifuged for 10 min at 8000 *g* in a Sorvall GSA rotor to remove a fine precipitate which formed during the rise in pH. The supernatant was concentrated by lyophilization to 30 mg of heparin/ml and the mixture was applied to a BioGel P10 chromatography system using two columns (5 cm $\times$ 128 cm) connected in tandem. The columns were packed and developed in 0.5 M NH₄CO₃ at a flow rate of 0.7 ml/min. Fractions (10 ml) were analysed for oligosaccharides by a carbazole procedure for quantification of uronic acid content [16]. The peak that emerged last from the column was a mixture of disaccharides. Each successive earlier peak was a mixture of tetra-, hexa-, octa-, deca-, dodeca- and larger oligo-saccharides. The mixture of disaccharides was further fractionated by gel filtration on a BioGel P2 column (1 cm $\times$ 60 cm) to obtain unsulphated, mono-sulphated and disulphated disaccharides. With the exception of the disulphated disaccharide $\Delta\text{U}_2\text{S}-(1-4)\text{-GINAc}_6\text{S}$ that was used in these experiments, all the other oligosaccharides were purified only according to size. Low-molecular-mass heparin (approx. 3000 Da) purchased from Sigma was used to compare activity with the different oligosaccharides.

Pg activation assays

Coupled assays were used to evaluate the initial rate of Pg activation by monitoring the amidolytic activity of generated Pm [17]. Pg was incubated at 37 °C in buffer containing 20 mM Hepes and 150 mM sodium acetate, pH 7.5, followed by addition of SK in clear plastic 96-well microtitre plates. Equimolar amounts of Pg and SK (20 nM) were used in all experiments. Pm activity was determined by hydrolysis of the substrate VLK-pNA (0.3 mM final concentration), monitored continuously at 405 nm in an Anthos Labtec Kinetic plate reader.

Initial velocities (V_i) were calculated from the slope ($b/2$) of plots of A_{405} versus t^2 using the equation $V_i = b(1 + K_m/S_0)/\epsilon k_{\text{cat}}$, where K_m is the apparent Michaelis constant for hydrolysis of

VLK-pNA by Pm (0.3 mM), S_0 is the initial concentration of VLK-pNA (0.3 mM), k_{cat} is the empirically determined catalytic rate constant for the hydrolysis of VLK-pNA by Pm ($1.35 \times 10^3 \text{ M} \cdot \text{min}^{-1} \cdot \text{mol of Pm}^{-2}$) and ϵ is the molar absorption coefficient of pNA at the wavelength 405 nm ($8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [18].

Inhibition constants for ATP, ATP[S] and the heparin polysaccharides were derived from Dixon plots of V_i/V_0 , where V_i and V_0 are the velocities of hydrolysis of VLK-pNA by Pm–SK in the presence and absence respectively of inhibitor, against different concentrations of the inhibitor ([I]). The intercept on the [I] axis gave a value of K_i for the different inhibitors [19].

Gel filtration chromatography

Separation of the complexes between SK and Pg from free reactants was accomplished by gel filtration on a Sephadex G-150 column (100 cm $\times$ 2 cm) equilibrated and eluted with 50 mM Tris/HCl, pH 7.5. Fractions of 1 ml were collected. ^{125}I -labelled human Pg was used to calibrate the column and the elution of the radiolabelled complexes was monitored by measuring radioactivity in a LKB 1272 γ -counter. ^{14}C - and ^{32}P -labelled complexes were measured in aliquots (50 μl) from each fraction in scintillation fluid (Packard; Insta-Gel) and counted in a Packard Tri-Carb 460 CD liquid scintillation counter using programs for ^{14}C and ^{32}P .

Assay of LTRPAHG SK peptide phosphorylation by the Pm–SK complex

Pm–SK (200 nM) phosphorylating activity was measured in 100 μl of a reaction mixture containing 50 μM ATP (1 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$), 500 μM calcium acetate, 500 μM magnesium acetate and 1 mM DTT in 50 mM Tris/HCl, pH 7.5. The reaction was started by adding different concentrations of ovalbumin or the LTRPAHG–ovalbumin conjugate and incubated for 20 min at room temperature. The reaction was stopped by adding 1 ml of 20% (w/v) trichloroacetic acid and the resulting precipitate was collected on 45 μm filter discs (Millipore). After washing with 3 ml of 20% trichloroacetic acid, the filters were transferred to vials containing scintillation fluid and radioactivity was counted in a Packard Tri-Carb 460 CD liquid scintillation counter using a program for ^{32}P .

Gel electrophoresis

Tricine/SDS gels (10%) were prepared as described by Schaeffer and Von Jagow [20]. The molecular mass standards used were β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14.4 kDa) and pancreatic trypsin inhibitor (6.5 kDa).

Protein sequence analysis

The proteins (100 pmol) were sequenced by automated Edman degradation in a gas/liquid phase sequencer (Model 477A; Applied Biosystems, Inc., Foster City, CA, U.S.A.) with on-line phenylthiohydantoin analysis using HPLC (Model 120A; Applied Biosystems). The instruments were operated as recommended in the user bulletins and manuals distributed by the manufacturer. Sequencing was performed in the Duke University Medical Center Comprehensive Cancer Center Macromolecular Shared Resource by Dr. Jan J. Engchild.

RESULTS

Effect of ATP on the amidolytic activity of Pm-SK

The effect of ATP on the amidolytic activity of the Pm-SK complex is shown in Figure 1. Equimolar amounts of Pg and SK (20 nM) were first incubated for 20 min followed by the addition of ATP or the non-hydrolysable analogue ATP[S]. Measurements of Pm activity and the calculation of the inhibition constants were done as described in the Experimental section. Both ATP and ATP[S] inhibited Pm-SK amidolytic activity, with K_i values of 14.2 and 11.23 mM respectively. Similar experiments were conducted with a variety of related nucleotides. Relative to the inhibition observed with ATP, ATP[S] and 3',5'-AMP, all other nucleotides tested had only a slight effect on Pm-SK amidolytic activity (Table 1).

Figure 1 Effects of ATP and ATP[S] on Pm-SK amidolytic activity

Increasing concentrations of ATP (●) or ATP[S] (○) were added to equimolar complexes (20 nM) of Pg and SK. The amidolytic activity of Pm was measured by monitoring the hydrolysis of the substrate VLK-pNA at 405 nm, as described in the Experimental section.

Table 1 Effect of nucleotides and bivalent cations on Pm-SK amidolytic activity

The concentration of nucleotides and bivalent cations used in each case was 500 μ M.

Nucleotide	$10^{-7} \times \text{Activity (mol of pNA/min)}$		
	Control	+ Mg^{2+}	+ Ca^{2+}
None	3.00	2.98	3.06
ATP	1.91	2.86	2.96
ATP[S]	0.98	0.93	1.08
3',5'-AMP	2.37	2.51	2.78
ADP	2.68	2.80	2.90
AMP	2.78	2.61	2.72
GTP	2.54	2.75	2.88
UTP	2.83	2.70	2.78
CTP	2.88	2.72	2.90

Figure 2 Reversal of the ATP-dependent inhibition of Pm-SK amidolytic activity by bivalent cations

Equimolar complexes of Pg-SK (20 nM) in the presence of ATP (500 μ M) or ATP[S] (500 μ M) were incubated with increasing concentrations of bivalent cations. ●, Pm-SK-ATP activity in the presence of Mg^{2+} ; ○, Pm-SK-ATP activity in the presence of Ca^{2+} ; ▲, Pm-SK-ATP[S] activity in the presence of Mg^{2+} ; △, Pm-SK-ATP[S] activity in the presence of Ca^{2+} .

Reversal of the ATP-dependent inhibition of Pm-SK amidolytic activity by bivalent cations

The ability of bivalent cations to alter the ATP-dependent inhibition of Pm-SK amidolytic activity was determined by preincubation of the preformed Pm-SK complex with ATP and ATP[S] in the presence of increasing concentrations of Ca^{2+} or Mg^{2+} . At concentrations greater than 200 μ M, both bivalent cations were able to overcome the inhibitory effect of ATP, whereas inhibition by ATP[S] was not reversed by these cations (Figure 2). This experiment suggests that the reversal of the inhibition in the presence of bivalent cations is related to hydrolysis of the γ -phosphate group of ATP, since inhibition by the non-hydrolysable analogue was not affected by the cations. Similar experiments conducted with related nucleotides are summarized in Table 1. These data show that all the other nucleotides affect the reaction only slightly either in the absence or in the presence of Mg^{2+} or Ca^{2+} .

We have previously demonstrated [20] that Ca^{2+} and Mg^{2+} inhibit the activation of Pg by catalytic amounts of Pm-SK. The IC_{50} value was 50 mM for each cation. However, using equimolar amounts of Pg and SK, the bivalent cations (500 mM) not only do not inhibit the activation of Pg in the presence of ATP, but stimulate the reaction (Table 1). A similar stimulation by these cations was previously observed on the activation of Lys⁷⁷-Pg by tissue-type Pg activator [21].

Effect of ATP on the Pg activator activity of the Pm-SK complex

Equimolar complexes of Pg1 and 2 with SK (0.2 nM) were used to activate either Pg1 or 2 (20 nM) with increasing concentrations of ATP in the absence or presence of Mg^{2+} . Table 2 shows that the catalytic activity of both Pm-SK1 and -SK2 was affected by ATP to the same extent (36% inhibition). As observed with the amidolytic activities of the Pm-SK complex, the inhibition by

Table 2 Effect of ATP and Mg^{2+} on Pm-SK plasminogen activator activity

Pg (20 nM) was activated in 20 mM Hepes, pH 7.4, at 37 °C with SK-Pm (0.2 nM) in the presence or absence of ATP (500 μ M) and Mg^{2+} (500 μ M) at constant ionic strength, as described in the Experimental section.

Enzyme	Substrate	Nucleotide	Cation	$10^{-11} \times$ Activity (mol of PM/min)
SK-Pm1	Pg1	None	None	2.765
SK-Pm1	Pg1	ATP	None	1.792
SK-Pm1	Pg1	ATP	Mg^{2+}	2.874
SK-Pm2	Pg2	None	None	2.803
SK-Pm2	Pg2	ATP	None	2.105
SK-Pm2	Pg2	ATP	Mg^{2+}	3.104

Table 3 Effect of glycosaminoglycans and magnesium on the amidolytic activity of Pm-SK

Compounds were present at 75 μ M and Mg^{2+} was present at 500 μ M.

Compound	Molecular mass (Da)	Mg^{2+}	$10^{-7} \times$ Activity (mol of pNA/min)
None	—	—	4.06 ± 0.014
Low-molecular-mass heparin	3000	—	1.81 ± 0.010
		+	4.88 ± 0.022
Heparin disaccharide	630	—	2.09 ± 0.020
		+	4.56 ± 0.017
Heparin hexasaccharide	1900	—	0.24 ± 0.20
		+	4.23 ± 0.018
Heparin octasaccharide	2500	—	0.74 ± 0.016
		+	4.63 ± 0.022
Heparin decasaccharide	3100	—	1.09 ± 0.030
		+	4.44 ± 0.021
Heparin dodecasaccharide	3800	—	0.76 ± 0.010
		+	4.36 ± 0.018

ATP could be reversed by addition of Mg^{2+} . The catalytic activity of the Pm-SK complex was only slightly affected (results not shown) by the nucleotides tested in Table 1.

Effect of heparin oligosaccharides on Pg activation by SK

It has been reported that ATP and heparin disaccharides with the basic structure $\Delta U,2S-(1 \rightarrow 4)\text{-GlcNS,6S}$, with a sulphate at the C-6 position of the glucosamine residue, possess a similar conformation [7]. Therefore we tested the capacity of heparin and related oligosaccharides to affect the amidolytic activity of Pm-SK (Table 3). Relative to the inhibition observed with the disaccharide, the magnitude of the inhibitory effect increased with the length of the oligosaccharide. The addition of Mg^{2+} not only reversed the inhibition caused by the heparin oligosaccharides, but it stimulated Pm activity. These data are consistent with previous results showing that, in the presence of heparin, both Pg activation by physiological activators and Pm activity are stimulated by Mg^{2+} [22]

Identification of the ATP binding domain of the Pm-SK complex

The experiments shown in Figure 3 were conducted to determine whether a ternary complex is formed between Pm-SK and ATP. All experiments were performed on a Sephadex G-150 column, as described in the Experimental section. Figures 3(a) and 3(b) show the chromatographic profiles of ^{125}I -labelled Pg and of an

Figure 3 Gel filtration chromatography of complexes between Pm-SK and ATP

A Sephadex G-150 column (100 cm $\times$ 2 cm) was used in all experiments, as described in the Experimental section. Fractions of 1 ml were collected. (a) Elution profile of ^{125}I -labelled Pg (20 nM). (b) Elution profile of an equimolar mixture (20 nM) of ^{125}I -labelled Pg and unlabelled SK. (c) Elution profile of a mixture containing Pg and SK (20 nM), $[^{14}\text{C}]\text{ATP}$ (1 μM , 0.4 μCi) and 1 mM magnesium acetate. (d) Elution profile of a mixture containing unlabelled Pg and SK (20 nM), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μM , 2 μCi) and 1 mM magnesium acetate.

equimolar mixture of Pg and SK respectively. Figure 3(c) shows the elution profile of PG-SK incubated with $[^{14}\text{C}]\text{ATP}$. Figure 3(d) shows the elution profile of PG-SK incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. These data demonstrate that the ^{32}P -containing peak in the Pm-SK complex (elution volume 18–35 ml) corresponds only to a phosphorylated complex, since the experiment conducted with $[^{14}\text{C}]\text{ATP}$ shows a single ^{14}C radioactive peak at elution volume 55–70 ml, corresponding to the elution of ATP from the column, as demonstrated in control experiments in which Pg or SK alone was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ prior to chromatography (results not shown).

Figure 4(a) shows the elution profile of an equimolar mixture of ^{125}I -labelled Pg (20 nmol) and unlabelled SK in the presence of 100 μM ATP[S]. A similar experiment was conducted by incu-

Figure 4 Gel filtration chromatography of Pg-SK-ATP[S], Pg-SK₁₇ and Pg-SK₁₇-ATP complexes

A Sephadex G-150 column (100 cm × 2 cm) was used in all experiments, as described in the Experimental section. Fractions of 1 ml were collected. (a) Elution profile of a mixture containing ¹²⁵I-labelled Pg (20 nM), unlabelled SK (20 nM) and ATP[S] (100 μM). (b) Elution profile of an equimolar mixture of ¹²⁵I-labelled Pg (20 nM) and unlabelled SK₁₇. (c) Elution profile of a mixture containing unlabelled Pg and SK₁₇ (20 nM), [γ-³²P]ATP (0.1 μM, 2 μCi) and 1 mM magnesium acetate.

bating ¹²⁵I-labelled Pg and unlabelled SK in the presence of 100 μM low-molecular-mass heparin. The elution profile (results not shown) was similar to the one shown in Figure 4(a). Figure 4(b) shows the chromatographic profile of an equimolar mixture of ¹²⁵I-labelled Pg (20 nmol) and unlabelled SK₁₇ comprising residues Val¹⁴³-Arg²⁸⁹. Figure 4(c) shows the elution profile of an equimolar mixture of unlabelled Pg (20 nmol) and SK₁₇ in the presence of [γ-³²P]ATP. These data demonstrate that ATP[S] and heparin do not prevent the formation of a complex between Pg and SK. In addition, we also demonstrate that the Val¹⁴³-Arg²⁸⁹ SK₁₇ fragment, although it forms a complex with Pg, is unable to phosphorylate the complex in the presence of [γ-³²P]ATP.

Analysis of the ³²P-labelled Pm-SK complex

Gel electrophoresis analysis of the ³²P-labelled Pm-SK complex isolated by gel filtration is shown in Figure 5(a). Several protein bands corresponding to the fragmentation of SK and Pm in the absence (Figure 5a, lane 2) and the presence (Figure 5a, lane 3) of [γ-³²P]ATP show no significant differences in the pattern of degradation of both Pm-SK complexes. Autoradiography of the ³²P-labelled complex shows a single radioactive peptide in the area of 4.5 kDa (Figure 5a, lane 4). Parallel samples were

Figure 5 Analysis of the ³²P-labelled Pm-SK complex

Protein samples were resolved on 10% Tricine/SDS gels under reducing conditions, as described in the Experimental section. (a) Lanes 1–3 show a Coomassie Blue-stained gel. Lane 1, molecular mass markers; lane 2, Pg (50 μg) after activation for 1 h by SK (30 μg); lane 3, ³²P-labelled Pm-SK isolated as described for Figure 3(d); lane 4, autoradiogram of the material in lane 3. (b) Structural position of the peptides identified in the Pm-SK complex.

transferred to Immobilon membranes and subjected to N-terminal sequence analysis by the Edman degradation method. Figure 5(b) shows a scheme depicting the origin of the different peptides within the Pm-SK complex analysed in Figure 5(a), lane 3. The fragments obtained consisted of: (1) a 67 kDa peptide with the N-terminal sequence G¹PLDDT, corresponding to the Pm heavy chain generated after Pg activation by equimolar amounts of SK; (2) one 27 kDa peptide with the N-terminal sequence V⁵⁶¹VGG, corresponding to the Pm light chain; (3) a 37 kDa SK peptide with the N-terminal sequence I¹AGPEW; (4) a 7 kDa peptide with the N-terminal sequence V³⁵⁴EDNH; and (5) a 42 kDa SK peptide with the N-terminal sequence S⁶⁰KP FAT; and (6) a 4.5 kDa peptide with the sequence I¹AGPEW. The only ³²P-labelled peptide found in the complex was the SK 4.5 kDa N-terminal region. Several of the SK polypeptides identified in the complex contained the I¹AGPEW sequence; however, none were labelled. These experiments suggest that the N-terminal region of SK must be hydrolysed, producing the 4.5 kDa fragment, prior to functioning as a substrate for its phosphorylation. Since the ³²P-labelled Pm-SK complexes were purified by gel filtration, these experiments suggest that even the cleaved SK polypeptides remain tightly bound to Pm as an integral part of the complex.

Determination of the phosphorylating activity of the Pm-SK complex

Examination of the primary amino acid sequence of the 4.5 kDa SK N-terminal region for possible sites of phosphorylation indicated that the sequence L⁴²TSRPAHG⁴⁹ conforms with sequences surrounding phosphorylated sites in many intracellular phosphoproteins [23]. That is, the phosphorylated amino acid (either serine or threonine) is, in general, separated from a basic amino acid (either lysine or arginine) by one amino acid. Since synthetic peptides have been used previously as substrates for protein kinases [24] we conjugated the peptide L⁴²TSRPAHG to ovalbumin and employed it as a substrate to detect the phosphorylating activity of the Pm-SK complex. Assays were conducted as described in the Experimental section. Figure 6 shows a gel electrophoresis analysis of the proteins

Figure 6 Analysis of the ^{32}P -labelled peptides resulting from Pm-SK phosphorylating activity

Protein samples were resolved on 10% Tricine/SDS gels under reducing conditions, as described in the Experimental section. Lanes 1–4 show a Coomassie Blue-stained gel; lanes 5 and 6 show an autoradiogram of the material in lanes 3 and 4 respectively. Lane 1, ovalbumin (20 μg); lane 2, LTSRPAHG-ovalbumin conjugate (20 μg); lane 3, Pg (10 μg) after activation for 1 h with SK (6 μg), then incubated with ovalbumin (20 μg) in the presence of 1 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; lane 4, as in lane 3, except that the substrate was LTSRPAHG-ovalbumin (20 μg).

phosphorylated by the Pm-SK complex. Autoradiography of the ^{32}P -labelled peptides show a minor radioactive band in the region of 45 kDa corresponding to the control with ovalbumin, and a strong band in the 4.5 kDa region (Figure 6, lane 5), suggesting that the SK peptide in the complex is the major substrate for phosphorylation under these conditions. However, when we used the LTSRPAHG-ovalbumin conjugate as a substrate, we observed strong signals in both the 45 and 4.5 kDa regions (Figure 6, lane 6), suggesting that the conjugate serves as a substrate for the phosphorylating activity of the Pm-SK complex. Results of the incubation of the Pm-SK complex with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Mg^{2+} and increasing concentrations of ovalbumin and the LTSRPAHG-ovalbumin conjugate are shown in

Figure 7 Assay of the phosphorylating activity of the Pm-SK complex

Equimolar amounts of Pg and SK (20 nM) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were incubated with increasing amounts of ovalbumin (○) or LTSRPAHG-ovalbumin (Ov.) conjugate (●) for 20 min at room temperature, as described in the Experimental section.

Figure 7. The conjugate was the preferred substrate, suggesting that the SK sequence LTSRPAHG contains a target site for the phosphorylating activity of the Pm-SK complex in the presence of ATP.

DISCUSSION

Pg activation by SK proceeds via a two-step mechanism. First, SK binds to Pg inducing a conformational change in Pg [25] which fully exposes the zymogen active site [2]. This is followed by autolytic cleavage of the internal bond $\text{Arg}^{560}\text{-Val}^{561}$ in Pg which converts the transient Pg-SK complex into Pm-SK, which then functions as a potent Pg activator [2], a property not present in either Pm or SK alone. Induction of proteolytic activity in Pg by SK does not require cleavage of the internal $\text{Arg}^{560}\text{-Val}^{561}$ bond in Pg [25,26]. In fact, Summaria et al. [25] demonstrated that the Pg-SK complex can be dissociated resulting in proteolytically active Pg, confirming the conformational change in Pg induced by SK. Both SK N-terminal and C-terminal regions, although cleaved, remain attached to the Pm-SK complex and are probably essential in maintaining a Pm conformation required for full Pg activator activity [27]. The $\text{Ser}^{60}\text{-Lys}^{333}$ SK peptide is essential for minimal activator activity, the $\text{Ala}^{334}\text{-Lys}^{414}$ C-terminal peptide is essential for high affinity with Pm and the $\text{Ile}^1\text{-Lys}^{59}$ N-terminal peptide is important for maintaining the proper conformation of SK if it is to demonstrate full activator activity [27]. Moreover, incubation of SK with Pg for periods longer than 2 h generates an SK peptide comprising residues $\text{Val}^{143}\text{-Arg}^{289}$ [15] which retains full Pg binding capacity, although its activator activity is minimal.

Based on a previous observation that ATP and 3',5'-AMP could inhibit SK activating function [5], we performed additional experiments to elucidate the mechanism of the ATP-dependent inhibition of Pm-SK. Our data demonstrate that ATP inhibits not only the amidolytic but also the Pg activator activity of the Pm-SK complex. The inhibition by ATP in both cases can be reversed by bivalent cations at concentrations greater than 200 μM . Moreover, at a concentration of 500 μM for each cation, there is a stimulation of Pg activation by SK. Previous work in our laboratory [21] demonstrated that Ca^{2+} and Mg^{2+} inhibit the activation of Glu-Pg by catalytic amounts of Pm-SK. However, when the substrate was Lys-Pg, the cations stimulated the activation of Pg [21]. The activation of Pg by equimolar amounts of SK proceeds via a conformational change in the Pg molecule [1]. Therefore cleavage of the internal bond $\text{Arg}^{560}\text{-Val}^{561}$ under these conditions should proceed via a mechanism where the substrate Glu-Pg would exist in an open conformation, similar to the conformation of Lys-Pg [28]. Since the conformation of the substrate is critical for the effect of the bivalent cations [21], this would explain the stimulation observed after reversal of the inhibition produced by ATP.

Previous studies have demonstrated that ATP and heparin oligosaccharides have a similar structural conformation [7]. Since heparin has been reported to inhibit the thrombolytic effect of SK [29], we investigated the effects of heparin and heparin-derived oligosaccharides on the activation of Pg by equimolar amounts of SK. In the absence of bivalent cations, heparin and its derivatives are potent inhibitors of the activation. However, addition of Mg^{2+} and Ca^{2+} at concentrations greater than 100 μM not only abolished the inhibition, but stimulated the reaction. The nature of the heparin effect on Pg activation depends upon the ionic strength [22]. At low ionic strength (< 25 mM) heparin acts as an inhibitor, at intermediate ionic strength (> 25 and < 200 mM) it acts as a stimulator and at high ionic strength (> 200 mM) it has no effect. Our experiments were conducted at

a constant ionic strength of 150 mM with added sodium acetate. Under these conditions, increasing concentrations of these bivalent cations produce a stimulation of Pg activation by activators in the presence of heparin [22]. Therefore our findings are consistent with these observations.

Molecular models [7] indicate that one of the possible conformations of the inhibitor Δ U,2S-(1 $\rightarrow$ 4)-GlcNAc,6S resembles part of the structure of ATP, showing the negative charge (SO^{3-}) and the sugar ring (Δ uronyl) of the disaccharide in the same plane as the PO_4^{4-} and ribose of ATP. Our experiments suggest that both molecules may bind to a similar site on SK which is formed after interaction with Pg, since neither ATP[S] nor heparin prevents the formation of the Pg-SK complex. Addition of bivalent cations should stimulate the hydrolysis of the γ -phosphate of ATP or neutralize the negative charges of heparin. The neutralization of the negative charges of the non-hydrolysable ATP[S] by bivalent cations is not enough to overcome the inhibitory effect of this compound and suggests that hydrolysis of the γ -phosphate is necessary to abolish inhibition. The site in SK is highly specific for ATP or heparin, because GTP, UTP, CTP or AMP are not able either to inhibit the activation of Pg by SK or to reverse the inhibition produced by heparin [30].

Although proteolytic or enzymic activity has not previously been associated with SK alone, it is reasonable to speculate that some changes in SK tertiary structure may occur after interaction with Pg, mainly if we consider the magnitude of the conformational change occurring in Pg when it is converted to Pm [28]. Our data suggests that Pm-SK in the presence of ATP and bivalent cations may function as an autophosphorylating enzyme, where the SK segment L⁴²TSRPAHG⁴⁹ is the target of this activity. We also demonstrate that this peptide serves as an *in vitro* substrate for the phosphorylating activity of the Pm-SK complex. The presence of the SK N-terminal region is necessary for the complex to function as a kinase. SK₁₋₇, which lacks this region, forms a complex with Pg but does not develop phosphorylating activity. Our data also suggest that phosphorylation must be preceded by proteolytic cleavage of the 4.5 kDa N-terminal region, which then remains linked to Pm-SK as an integral part of the complex.

ATP is not required for Pm-SK to generate the 4.5 kDa N-terminal peptide. However, ATP may regulate proteolysis and the resulting phosphorylation of this segment. Plasma concentrations of ATP range between 480 and 980 μM [31]. Therefore it is interesting to speculate that thrombolytic therapy with SK may be affected by this mechanism. Similarly, heparin has been reported to inhibit thrombolysis by SK [27]. Since both molecules apparently bind to the same site in SK, heparin administration prior to thrombolytic therapy with SK should be questioned. ATP binding to Pm-SK should require an ATP binding pocket. The ATP binding consensus sequences found in proteins known to form a nucleotide binding pocket [32] are a β -sheet-Gly-Lys-Thr-loop, an α -helix in domain A and an α -helix-loop Φ_4 - β -sheet in domain B. SK structural analysis predict a possible A domain containing a β -sheet-Gly-Lys-Thr-loop followed by an α -helix between residues 50–60, as determined by Chou–Fasman calculations [33,34], and a possible B domain with an α -helix-loop Φ_4 - β -sheet between residues 70–90 [34]. Identification of the ATP binding pocket in SK requires structural analysis, which will be the subject of future studies in our laboratory.

It was previously reported that ATP may serve as a specific activator of the cathepsin D cleavage of parathyroid hormone in the kidney [6]. In the case of the proteinase Pm-SK, ATP-driven

phosphorylation of SK may serve Streptococci in a variety of mechanisms where phosphorylation of ligands may originate signal transduction mechanisms related to bacterial attachment to mammalian cells [35]. In this context, we have previously found that SK can bind to human synovial fibroblasts in culture not only as a complex with either Pg or Pm, but also via a separate binding site on the cell surface which recognizes fibronectin as a ligand [36]. Interestingly, the capacity of SK to compete for fibronectin binding sites in these cells is due to sequence similarities between fibronectin and the SK N-terminal region [37]. We also demonstrated that a fibronectin fragment containing the region with the SK sequence similarity is an inhibitor of Pg activation by SK [37]. Therefore the potential pathogenicity of this novel mechanism cannot be underestimated.

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