Characterisation of the Sek-1 receptor tyrosine kinase

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Abstract **We present an initial characterisation of the mouse Sek-1 protein, a member of the Eph subfamily of putative receptor tyrosine kinases, which has been proposed to play a role in the segmentation of both the hindbrain and the mesoderm. Antibodies raised against the protein have been used to confirm the early embryonic expression pattern previously established by mRNA in situ hybridisation. These antibodies, together with the expression of the *Sek-1* gene in a baculovirus system, were instrumental in demonstrating that the protein carries a tyrosine kinase activity and that it is presented at the cell surface with its N-terminal (putative ligand-binding) domain outside of the cell. Therefore, as expected from its amino acid sequence, Sek-1 conforms to the general model of receptor-type tyrosine kinases.**

Key words: Receptor tyrosine kinase; Baculovirus; *Eph*; *Sek-1*; Hindbrain segmentation

1. Introduction

Receptor-tyrosine kinases (RTKs) are transmembrane proteins mediating the transduction of signals required for the control of various and essential developmental processes, such as cell growth, differenciation and migration [1]. In all cases analysed so far, ligand binding leads to RTK dimerisation and activation of the cytoplasmic tyrosine kinase catalytic domain resulting in receptor autophosphorylation at specific tyrosine residues [2]. Subsequently, phosphorylated receptors associate with a specific repertoire of cytoplasmic kinase substrate proteins that belong to different signalling pathways [3].

The recently discovered Eph family has rapidly grown to represent the largest RTK subfamily [4,5]. Its members are characterised by a specific type of putative extracellular domain which includes a series of 19 conserved cysteine residues and two fibronectin type III repeats. The catalytic intracellular domain and the C-terminal extremity are also highly conserved among the different members. The presence of the fibronectin type III motifs, which are observed in several adhesion molecules [6,7], raises the possibility that Eph family members might be involved in cell–cell or cell–matrix interactions in addition to their possible role in signal transduction. However, the biochemical and functional characterisation of the Eph family has so far been limited. Despite the analysis of the developmental expression pattern of a few members [5,8–21] and the recent cloning of genes encoding candidate ligands [22–31], the mode of action of these proteins, the elements of their downstream transduction pathways and their biological functions are largely unknown, although an interaction between one member (Eck) and phosphatidylinositol-3 kinase has recently been reported [32], and Eck's ligand (B61) has been shown to be involved in angiogenesis [33].

In this study, we present an inital characterisation of the Sek-1 protein. We have raised antibodies against Sek-1 and demonstrate that they reveal a pattern in whole mount mouse embryos consistent with previous mRNA in situ hybridisation studies. We have used these antibodies in conjunction with the production of Sek-1 in a baculovirus expression system to analyse the subcellular localisation of the protein and to demonstrate that it is phosphorylated on tyrosines in vivo, and that in vitro it has the capacity for autophosphorylation on tyrosine residues.

2. Materials and methods

2.1. Production of antibodies and immunohistochemistry

The Sek-1I antibody, directed against the intracellular part of Sek-1, was obtained by injection of a peptide corresponding to the 11 C-terminal amino-acids of Sek-1 coupled to Keyhole-Limpet hemocyanin (KLh). For generation of antibodies directed against the extracellular part of Sek-1 (Sek-1E), an MS2-polymerase fusion protein including the N-terminal part of Sek-1 (nucleotide positions 258 to 879) was produced in bacteria. Rabbits were injected every three weeks with 10 μg of KLh-peptide (6 injections), or 50 μg of the MS2-polymerase-Sek-1 fusion protein (5 injections). Immuno-histochemistry on whole mount embryos was performed as described [34]. The anti-Sek antibodies and the peroxidase-conjugated goat anti-rabbit antibody (Sigma) were added at 10000- and 200-fold dilutions, respectively. The peroxydase revelation was performed using 2,5 g/l 4-chloro-1-naphtol in a 40% ethanol solution containing 0,006% v/v H_2O_2.

2.2. Expression of Sek-1 using the baculovirus system

A *Sek-1* cDNA fragment (nucleotide positions 57 to 3198) was subcloned into the pBluebac vector (Invitrogen). The resulting plasmid was cotransfected with a wild-type baculovirus to produce a *Sek-1* recombinant baculovirus, which was selected by a revelation of β-galactosidase activity and hybridisation with a *Sek-1* cDNA probe. Spodoptera frugiperda (Sf9) cells (kindly provided by Lucien Cabanié) were cultured in TC-100 medium (Gibco), supplemented with 10% fetal bovine serum (Techgen), 100 u/ml penicillin, 100 mg/l streptomycin, 100 mg/l kanamycin and 2 mM glutamine (Gibco). Infections were performed on plates as described [35].

2.3. Immunofluorescence and confocal microscopy

48 hours after infection, cells were preincubated in PBS, 0,5% BSA; Sek-1I or Sek-1E antibodies were added at a 7500-fold dilution, for 1.5 hours. Following 2 × 5 minutes washes in the preincubation solution, a FITC-conjugated anti-rabbit antibody (Biosys) was added at a 300-fold dilution and incubated for one hour. After two additional PBS washes and fixation on cover slides coated with poly-L-lysine (Sigma) in a 1:1 methanol/acetone solution at 4°C, slides were covered and examined with a confocal microscope as described [36].

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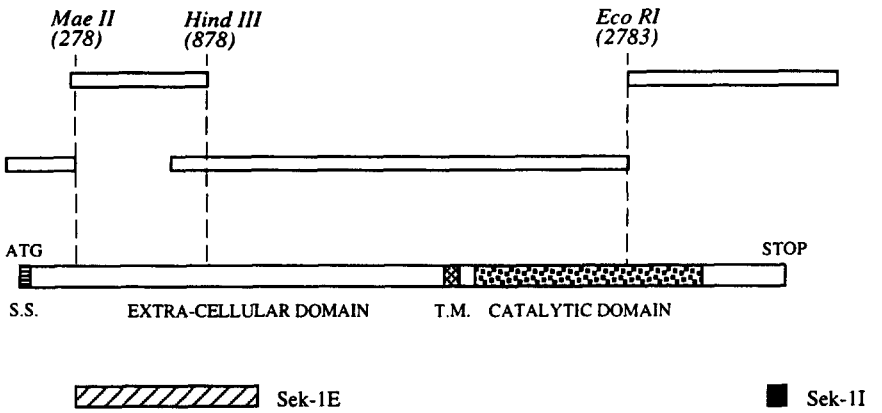


Fig. 1. Schematic representation of the cDNA clones used for the reconstruction of a full-length cDNA sequence of *Sek-1*, and of the regions encoding the different domains of the protein. The regions of the protein used to raise the Sek-1E and Sek-1I antibodies are indicated underneath. Restriction sites and nucleotide positions are indicated in italics. S.S, signal sequence; T.M., transmembrane domain.

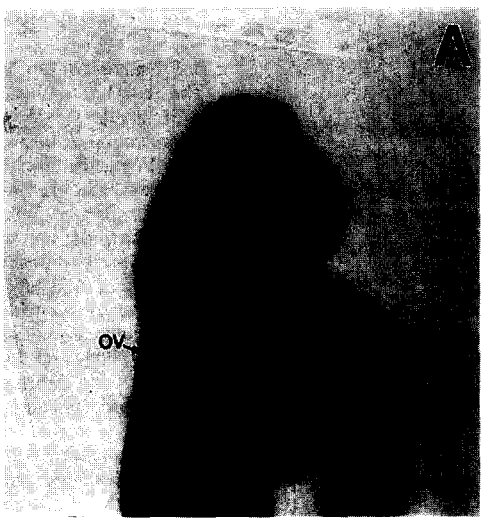
2.4. Immunoblotting, immunoprecipitation and kinase assay

Proteins were fractionated by electrophoresis on 7,5% polyacrylamide gels. Quantification was carried out by Coomassie blue staining and comparison with β-galactosidase used as a marker. Fractionated proteins were transferred onto nitrocellulose filters (Schleicher and Schüll), and immunodetection was performed using a chemiluminescence kit (Boehringer) as recommended by the manufacturer. Rabbit polyclonal anti-Sek-1 and mouse monoclonal anti-phospho-tyrosine (Sigma) antibodies were used at 7500- and 2000-fold dilutions respectively. Peroxidase-conjugated secondary antibodies were used at 10000- and 2000-fold dilutions for the anti-rabbit (Bio-Rad) and the anti-mouse (Institut Pasteur) antibodies, respectively. Immunoprecipitations were performed with the Sek-1I antibody conjugated to Protein-A Sepharose beads (Pharmacia) as recommended by the manufacturer. Cell extracts and kinase assays were performed as described [37].

3. Results

3.1. Reconstruction of the complete Sek-1 coding sequence and generation of specific antibodies

A cDNA covering the complete coding sequence of the *Sek-1*



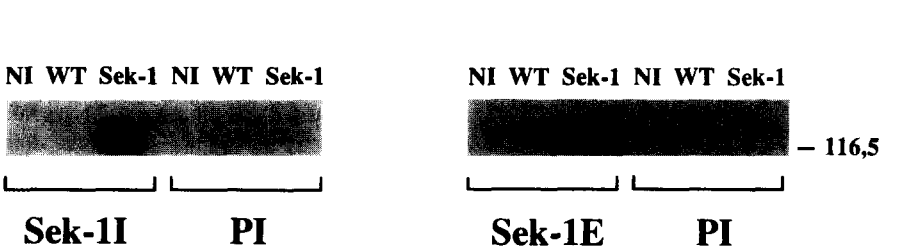


Fig. 2. Characterisation of the antibodies directed against Sek-1. (A) Whole mount immunodetection of the Sek-1 protein with the Sek-1I antibody. Dorsal view of a 9.5 dpc mouse embryo, showing Sek-1 in rhombomeres 3 (r3) and 5 (r5) and in the otic vesicle (ov). (B) Western blot detection of Sek-1 from baculovirus infected insect cells. For each lane approximatively 10^4 Sf9 cells were lysed in sample buffer, deposited on a 7.5% polyacrylamide gel and analysed by western blotting using either the Sek-1I, Sek-1E or pre-immune (PI) seras. NI, non-infected cells; WT, wild-type baculovirus infected cells; Sek-1, Sek-1 baculovirus infected cells.

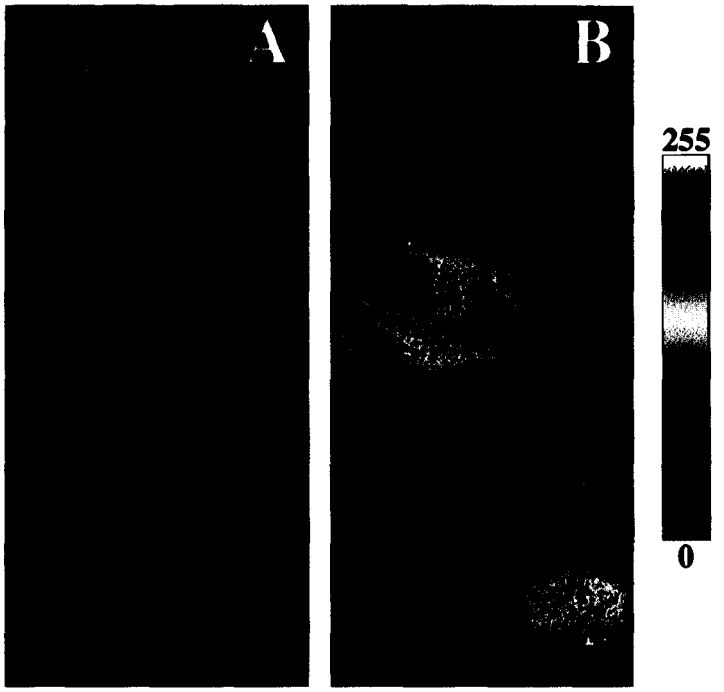


Fig. 3. Sek-1 incorporation into the cell membrane. Cells infected with the Sek-1 baculovirus were incubated at 4°C with Sek-1I (A) or Sek-1E (B) antibodies, processed for immunodetection with a fluorescence- conjugated second antibody, and observed in confocal microscopy. Labelling is observed only with Sek-1E, directed against the putative extracellular domain. A linear colour scale (0–255) is shown.

gene was obtained by ligation of four previously isolated fragments [9] (Fig. 1). The *Sek-1* cDNA encodes a protein with a predicted unmodified molecular mass of 107279 Da [9]. Consistently, in vitro translation of *Sek-1* mRNA derived from the cDNA clone generated a 110 kDa protein (data not shown).

Rabbit polyclonal antibodies were generated against a peptide consisting of the 11 carboxy-terminal amino acids and a bacterial fusion protein, including a large part of the putative extracellular domain. They were named Sek-1I and Sek-1E respectively. Their specificity was analysed by immunohistochemistry on whole mount 9.5 day post-coitum embryos. In both cases labelling of rhombomeres 3 and 5, of the otic vesicle, and of the prospective somites, was observed (Fig. 2A and data not shown). This is consistent with the previously reported mRNA pattern [9,10]. No labelling was observed with pre-immune sera (data not shown). These data demonstrate the specificity of the Sek-1E and Sek-1I antisera.

3.2. *Characterisation of the Sek-1 protein produced in a baculovirus expression system*

To analyse the cellular localisation of the Sek-1 protein, as well as its possible kinase activity, we used a baculovirus expression system to produce large amounts of protein, with presumably bona fide post-translational modifications [38]. A recombinant baculovirus, containing the coding sequence of *Sek-1* under the control of the viral polyhedrin promoter, was constructed. Western blotting analysis of extracts from cells infected with the Sek-1 recombinant baculovirus led to the detection of a 120 kDa protein with both Sek-1E and Sek-1I antibodies (Fig. 2B). The 120 kDa protein was not observed with non-infected cells or cells infected by a wild-type virus, nor was it detected with pre-immune sera (Fig. 2B). The amount of Sek-1 protein produced was estimated to about one microgram per 2×10^5 Sf9 cells (data not shown). The antibodies were subsequently used to study the cellular localisation of Sek-1 by immunofluorescence analysis of infected cells. Sf9 cells infected with the recombinant Sek-1 baculovirus produced very large amounts of Sek-1 protein, most of which accumulated within the cytoplasm (data not shown). To demonstrate that receptor molecules were expressed at the cell surface, infected cells were incubated with Sek-1E or Sek-1I antibodies without permeabilisation. As expected, no staining was observed with the Sek-1I antibody (Fig. 3A). In contrast, strong surface labelling was observed with Sek-1E antibody (Fig. 3B). These data demonstrate that at least part of the Sek-1 molecules present in Sf9

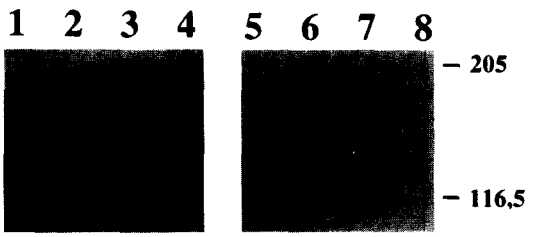


Fig. 4. Sek-1 is phosphorylated on tyrosine residues in vivo. Extracts from approximatively 5×10^4 Sf9 infected cells were immunoprecipitated with the Sek-1I antibody and subjected to Western blot analysis. Lanes 1 and 5 correspond to extracts prepared from wild type baculovirus infected cells, while the other samples were obtained from Sek-1 recombinant baculovirus infected cells. In lanes 2 and 6, the immunoprecipitation was performed in presence of a competitor peptide (0.2 mg/ml) while in the other lanes no competitor was included. In lanes 4 and 8, the immunoprecipitation was performed with the preimmune antibody. The Western blot was revealed with an anti-phosphotyrosine antibody in the lanes 1 to 4. The same blot was subsequently stripped and incubated with the Sek-1E antibody (lanes 5 to 8). The positions of migration of molecular mass markers (in kDa) are indicated.

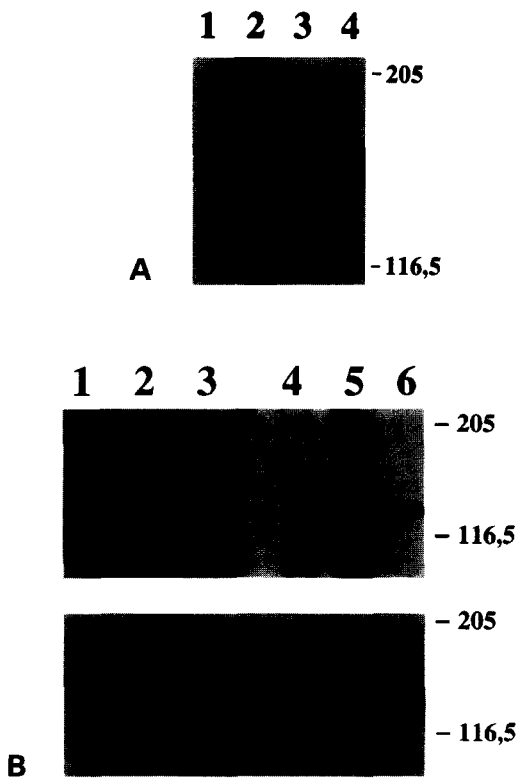


Fig. 5. Autophosphorylation of Sek-1 on tyrosine residues. (A) In vitro phosphorylation of Sek-1. Extracts from approximatively 5×10^4 Sf9 infected cells were immunoprecipitated with the Sek-1I antibody, incubated with [γ-^{32}P]ATP, separated by electrophoresis on a 7.5% polyacrylamide gel and revealed by autoradiography. Lane 1 corresponds to extracts prepared from wild type baculovirus infected cells, while the other lanes are obtained from Sek-1 recombinant baculovirus infected cells. In lane 3, the immunoprecipitation was performed in presence of a competitor peptide (0.2 mg/ml) which was omitted in the other lanes. In lane 4, the immunoprecipitation was performed with the preimmune sera. (B) Western blot analysis of autophosphorylated Sek-1 with anti-phosphotyrosine antibodies. Extracts from Sf9 cells infected with the Sek-1 recombinant baculovirus (except for lanes 1 and 4, in which case the cells were infected with wild-type virus) were immunoprecipitated with the Sek-1I antibody. They were subsequently incubated with (lanes 1, 3, 4 and 6) or without (lanes 2 and 5) [γ-^{32}P]ATP, separated by electrophoresis on a 7.5% polyacrylamide gel and blotted. In the upper part of the figure, the filters were analysed with either anti-phosphotyrosine (lanes 1 to 3) or Sek-1E (lanes 4 to 6) antibodies. In the lower part, the same filters were exposed for autoradiography (two days). Habitually, the equivalent of approximatively 2×10^5 cells was deposited per lane, except for lanes 2 and 5 where approximatively 10^6 cells were used. The positions of migration of molecular mass markers (in kDa) are indicated.

cells are incorporated into the cytoplasmic membrane with the expected orientation, i.e. with the N-terminal domain exposed outside.

3.3. Sek-1 is phosphorylated on tyrosine residues in Sf9 cells

The phosphorylation status of Sek-1 synthetised in Sf9 cells was analysed in the following way. Extracts from infected cells were submitted to an immunoprecipitation with the Sek-1I antibody, followed by an analysis by Western blotting. The blots were first revealed with an anti-phosphotyrosine antibody. After stripping, the blots were reprobed with the Sek-1E antibody. Both antibodies led to the detection of a single band, corresponding to the same mobility (Fig. 4). The corresponding protein was not detected from wild-type virus infected cell extracts. These data demonstrate that at least part of the Sek-1 molecules in Sf9 cells are phosphorylated on tyrosine residues.

3.4. Sek-1 auto-phosphorylation on tyrosine residues in vitro

Although the tyrosine phosphorylation of Sek-1 indicates its correct post-translational modification in the baculovirus expression system, it does not however demonstrate the phosphorylation activity of the protein. To investigate such an activity, we performed a kinase assay on immunoprecipitated Sek-1 molecules. We found that incubation of the Sek-1 immunoprecipitate with [γ-^{32}P]ATP led to labeling of the protein (Fig. 5A), suggesting that autophosphorylation indeed occurs. To demonstrate that this autophosphorylation involves tyrosine residues, the immunoprecipitated protein was subjected to the kinase assay in presence or in absence of ATP (Fig. 5B, lower part) and analysed by Western blotting using the anti-phosphotyrosine antibody. The phosphotyrosine signal was approximatively 5 times more intense in the presence of ATP, relative to the amount of Sek-1 deposited (Fig. 5B, upper part). Sek-1E antibody was used to confirm that the tyrosine-phosphorylated and [γ-^{32}P]ATP-labelled protein corresponded to Sek-1. Together, these data demonstrate that the Sek-1 protein can modulate autophosphorylation on tyrosine residues.

4. Discussion

The characterisation of the Sek-1 protein presented in this paper relied on the generation of two specific antibodies, directed against the N- and C-terminal domains, and on the production of Sek-1 proteins in a baculovirus expression system. The antibodies allowed us to demonstrate that the distribution of the protein in 9.5 day post-coitum mouse embryos is consistent with the previously described mRNA pattern. The immuno-localisation of Sek-1 in insect cells expressing the mouse protein established that it is anchored at the cellular membrane, with its C-terminal domain inside the cell and its N-terminal domain outside. Finally, we showed that Sek-1 prepared from these cells is phosphorylated on tyrosine residues and that it has the capacity to autophosphorylate on tyrosine residues in vitro. Consistent with our data, the putative chick ortholog of Sek-1, Cek-8, is recognised by antibodies directed against phosphotyrosine residues [39].

These results support the hypothesis, based on the examination of the Sek-1 amino acid sequence [9], that this molecule might constitute a membrane-bound receptor protein tyrosine kinase. They suggest that Sek-1 conforms to the general model for receptor tyrosine kinases which implies the presentation of the putative ligand binding domain at the external surface of the cell and the presence of the catalytic domain inside. Furthermore, the Sek-1 tyrosine autophosphorylation activity is consistent with receptor autophosphorylation following dimerisation induced by ligand binding [2] or fixation of antibodies. The fact that some of the Sek-1 molecules are found phosphorylated on tyrosine residues in insect cells, although we do not know whether this involves the same residue(s) autophosphorylated in vitro , might be due to the presence of a ligand in the serum used for cell culture or to the very high concentration of the protein at the membrane [40]. Recently, a membrane-bound protein with affinity for the N-terminal

domain of Sek-1 was identified [24]. It therefore constitutes a putative ligand, although the relatively low measured affinity makes the physiological significance of this observation questionable. In any case, taken together these data establish that *Sek-1* indeed encodes a receptor-type tyrosine kinase which may play a role in signal transduction mechanisms involved in particular in central nervous system patterning during early development.

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