IDENTIFICATION OF A NOVEL HUMAN SAND FAMILY PROTEIN IN HUMAN FIBROBLASTS INDUCED BY HERPES SIMPLEX VIRUS 1 BINDING

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Summary. – Studies on interaction between Herpes simplex virus 1 (HSV-1) and human fibroblasts KMB-17 have demonstrated that HSV-1 binding to the cell surface could induce a specific gene response. In this study, the HSV-1 stimulation-related gene 1 (HSRG1), a new so far unknown gene function of cellular response induced by a specific stimulation with HSV-1, was cloned from the cDNA library established from mRNA of early gene response of KMB-17 cells. The gene product consisted of 547 amino acids and had a significant homology in six eukaryotic species. On the basis of its structure it was identified as a member of the SAND protein family. The HSRG1 protein was fused with glutathione S-transferase (GST) and expressed in *Escherichia coli* DHPα strain under the control of T7 promoter. An antibody to HSRG1 raised in mice was used to detect expression of the HSRG1 protein in KMB-17 cells stimulated by HSV-1 by an immunoprecipitation assay. It was found that the HSRG1 protein was induced in these cells by HSV-1 at high level.

Key words: Herpes simplex virus 1; SAND protein family; signal transduction

Introduction

Cellular signal transduction, which transfers the information of external stimuli to cell nuclei and induces the response of cells, not only performs functions in physiological response, but also contributes to some extent to the infection of virus to cells (Mossman *et al*., 2001; Borgland *et al*., 2000). Some data indicate that the initiated viral infection of cells after virus binding to cell receptors induces the signal transduction in cells (Raftery *et al*., 1999; Mondal *et al*., 2001). This is due to the fact that the virus binding to cell receptors actually mimics the interaction of a ligand with its receptor. The virus binding to its specific receptors distributed in cellular membrane is supposed to induce different gene response and, probably, different protein molecules' expression in cells (Tal-Singer *et al*., 1998; Boyle *et al*., 1999). Some earlier studies have reported that these protein molecules isolated from cells infected with viruses were probably functioning in a pathological process of virus infection. Therefore, to investigate these new protein molecules it is interesting to study the interaction between viruses and cells.

HSV-1 is a highly contagious pathogen, which can disturb a range of host cellular processes by interference with signal transduction (Scott *et al*., 2001). E.g., mitogenic kinase cascades are activated to establish a cellular environment favorable for virus replication (Halford *et al*., 1995; McLean *et al*., 1999). Apoptosis pathways are repressed during infection to prevent cell death prior to virus release and possibly to protect potential infected neuronal cells from injury (Chou *et al*., 1992; Leopardi *et al*., 1997). It is reported that many kinds of gene activations are induced specifically by HSV-1 binding to corresponding receptors on the cell surface.

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Abbreviations: EST = expression sequence tag; GST = glutathione S-transferase; HSRG1 = HSV-1 stimulation-related gene 1; HSV-1 = Herpex simplex virus 1; IPTG = isopropyl-β-D-thiogalactopyranoside; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of SDS

In investigating further this process, we established a cDNA library of the mRNA extracted from human fibroblasts KMB-17 with HSV-1 bound to receptors on cell surface. Based upon fragments with expression sequence tag (EST) indicating differences in differential mRNA display between expression in control and stimulated cells, a full-length cDNA of 1,748 bp designated HSRG1 (GenBank Acc. No. AF442486) was isolated and identified. The protein encoded by this gene showed a high identity with the SAND protein family. Putative function of SAND proteins is so far unknown except involvement in cellular signal transduction pathways.

Materials and Methods

cDNA library was constructed in pUC18 with the mRNA isolated from human fibroblasts KMB-17 infected with HSV 1 for 1 hr. Double-stranded cDNA synthesis was performed from 2–3 μg mRNA by reverse transcription using the SMART cDNA Library Construction Kit (Clontech) according to the manufacturer's instructions. After cDNA size fractionation, cDNAs longer than 500 bp were ligated with an *Eco*RI adaptor and cloned into the digested pUC18 vector. *E. coli* DH5α cells were transformed with this construct, plated on 100 mm LB agar plates with 100 μg/ml ampicillin (200 μl per plate) and incubated at 37°C overnight. Individual resistant clones were cultured overnight in LB broth with 100 μg/ml ampicillin, and the plasmids were purified from them using the Plasmid Purification Kit (Takara).

HSRG1 gene cloning. The amplified EST fragment that showed a difference in the differential mRNA display between cells with bound HSV-1 and control was used as the probe to screen the library in a hybridization test performed according to the standard protocol (Kawamura *et al.*, 2001), in which plasmids from the cDNA library were dotted on nylon membranes and hybridized with [α-^{32}P]dATP-labeled probe in the buffer composed of the 10x Denhardt's solution (0.2% bovine serum albumin, 0.2% Ficoll, and 0.2% polyvinylpyrrolidone), 1 mol/l sodium choride, 0.05 mol/l Tris-HCl pH 7.4, 10 mmol/l ethylenediaminetetraacetate (EDTA), 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured salmon sperm DNA at 65°C for 16 hrs. The hybridized membrane was washed twice with 0.1x SSC and 0.1% SDS at 65°C for 30 mins and autoradiographed. The positive clone was subjected to sequence analysis (Sanger *et al.*, 1977)

Sequence analysis. Nucleotide and deduced amino acid sequence comparisons were carried out using the BLAST 2.0 program at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the GenBank, EMBL, and SWISS-PROT databases. ORF analysis was performed on the obtained fragments using the OMIGA 2.0 software. The electronic localization of the chromosome was determined in Public Database of Human Genome Sequence (http://www.genome.ucsc.edu). Motif analysis was done by the Motif Scan program (http://us.expasy.org/prosite/) and the subcellular localization was performed by PSORT II sever (http://psort.nibb.ac.jp/).

Northern blot analysis. Total mRNA isolated from various human tissues using the mRNA Prep-Kit (Pharmarcia) was denatured by formaldehyde, separated on 1% agarose gel and blotted to PVDF membrane (Ausule *et al.*, 1998). A 572 bp fragment amplified by polymerase chain reaction (PCR) and labeled with [^{32}P]dATP was used as probe. Hybridization was performed in 2x SSC plus 0.1% SDS at 68°C overnight (16 hrs). After washing with 2x SSC and with 0.1x SSC plus 0.1% SDS twice at room temperature, respectively, the blot was dried and autoradiographed. β-actin served as control.

Subcellular localization of HSRG1 protein. The coding region of HSRG1 gene was amplified with the upstream primer P1 (5'-G CGA ATT CTG ATG GAG GTC GGA GGA GAC-3') and the downstream primer P2 (5'-T CGG AAT TCC GAG TCC AGT GAA CAA GCC-3'), and cloned into the fluorescent expression vector pEGFPN1 at the *Eco*RI site. The constructed expression vector pEGFP-HSRG1 was identified by restriction analysis and sequencing and transfected into KMB-17 cells using LIPOFECTIN^{R} according to the manufacturer's instructions. After growth at 37°C for 48 hrs the transfected cells were checked microscopically for green fluorescence originating from the HSRG1 protein fused with a fluorescent protein.

Expression of the HSRG1 protein and production of its antibody. The coding region of the HSRG1 gene obtained by PCR using the upstream P1 (5'-GC GAATTC ATG GAG GTC GGA GGA-3') and downstream primer P2 (5'-TCG GAATTC TCA GAG TCC AGT GAA C-3'), was transfected into the *Eco*RI site of the prokaryotic expression vector pGEX-5x-1. According to the protocol described earlier by Smith and Johnson (1988), the HSRG1 protein fused with GST was induced in *E. coli* BL21 strain with 1 mmol/l isopropyl-β-D thiogalactopyranoside (IPTG) in 2x YT medium at 37°C. After separation by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and isolation from the gel the HSRG1 fusion protein was purified by affinity chromatography on Glutathione-Sepharose 4B (Pharmacia) according to the manufacturer's instructions. A specific antibody against HSRG1 (the HSRG1 antibody) was raised in a Swiss mouse (18–20 g) immunized subcutaneously with the purified HSRG1 protein (100 μg/ mouse) mixed with equal amount of Freund's adjuvant, followed by a booster 4 weeks later.

Immunoprecipitation of HSRG1 protein was performed according to Sambrook *et al.* (1989). KMB-17 cells were grown in DMEM with 5% of FCS to 80–90% confluence at 37°C followed by incubation in methionine-free MEM or phosphate-free MEM at 37°C for 1 hr. Labeling was performed with the same media containing [^{35}S]methionine or [^{32}P]phosphate at 37°C for 2 hrs. At 1 hour after addition of virus inoculum to the cells they were labeled, rinsed twice with 10 ml of PBS and scraped in 100 μl of the RIPA buffer (150 mmo/l NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/l Tris-HCl pH 7.5), and thawed-frozen 3 times. After centrifugation at 12,500 rpm for 10 mins at 4°C, the supernatant was incubated with the HSRG1 antibody in RIPA buffer at 37°C for 1 hr and the A protein-Sepharose 4B (Sigma) was added for further incubation at 4°C for 1 hr. After washing 3 times with the RIPA buffer and centrifugation as above, the pellet of the A protein-Sepharose 4B absorbed immune complex was incubated in a sample buffer (2% SDS, 62.5 mmol/l Tris, 10% glycerol, 2% 2-mercaptoethanol pH 6.8) at 100°C for 5 mins. After centrifugation as above, the supernatant was subjected to SDS-PAGE, dried and autoradiographed.

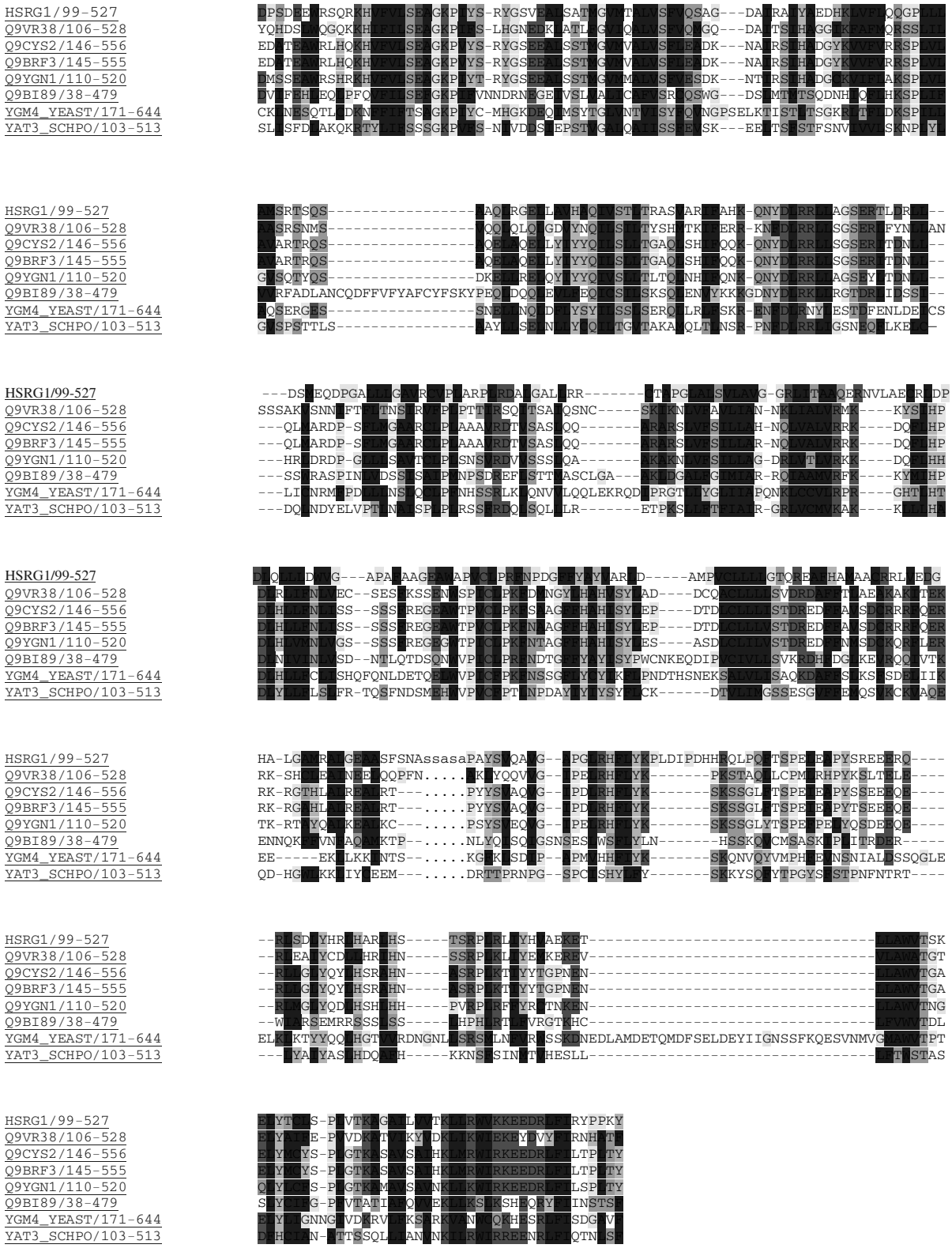

Fig. 1
Homology between the HSRG1 protein and seven SAND family proteins
Identical and similar amino acids are shaded.

Results

Identification of human HSRG cDNA

Screening of the cDNA library with an EST fragment used as probe for *in situ* hybridization and the sequence analysis revealed a 1,748 bp cDNA fragment occurring in positive clones. This fragment consisted of a coding region of 1,641 bp, a typical 5'-noncoding sequence and a 3'-noncoding sequence (Genbank Acc. No. AF442486). The deduced protein encoded by ORF of this gene had M_r of 59.2 K, consisted of 547 amino acids and had a calculated pI of 5.88. Following an extensive BLASTN search a homologue gene was found in human brain tissue. The structural analysis of the deduced HSRG1 protein in BLASTP searching revealed a family of a domain of high homology in at least 6 species, namely in human, mouse, *Fugu rubripes, Drosophila melanogaster, Caenorhabditis elegans*, and yeast (Fig. 1). This domain was composed of 513–556 amino acids. The homology between the compared domains was 24%–48%; an evolutionarily conserved domain was located at the C-terminal of all members of this family. For the time being, no data of its physiological function in cells have been reported except being named SAND proteins. Nevertheless, the genetic relationship between the HSRG1 protein and SAND proteins, showed in Fig. 2, implies a potential significance in cell biology.

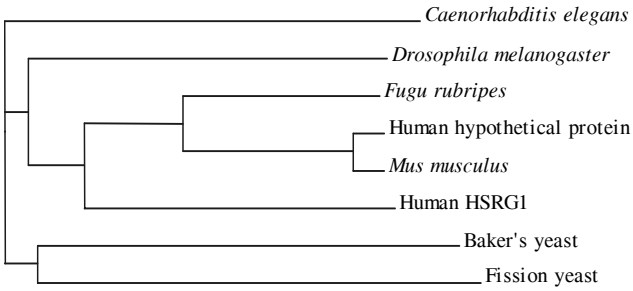

Fig. 2

Dendogram of the SAND protein family

Chromosome location of the HSRG1 gene

A comparison of the HSRG1 cDNA sequence with the Public Database of Human Genome Sequence revealed that the HSRG1 gene was located in the chromosome16q23.1 and contained 5 exons ranging in size from 148 bp (exon 4) to 830 bp (exon 3), encompassing genomic DNA of approximately 7 kbp. The exon sequence exhibited 99% identity with the HSRG1 cDNA. The exon-intron boundaries were confirmed as consensus splicing signals in which AG and GT dinucleotides were always found to occur at the splice acceptor and donor site, respectively.

Tissue distribution of HSRG1 mRNA

To investigate the distribution of HSR1 mRNA in tissues Northern blot analysis of poly A^+ RNA extracted from human tissues including brain, heart, liver, skin, pancreas, lung, kidney, and skeletal muscle was performed with a labeled 572 bp cDNA fragment of HSRG1 gene as probe and the results indicated that the HSRG1 gene had only one kind of transcript of 4.2 kb in 6 of 8 tissues under study. No expression of the HSRG1 gene in the pancreas and kidney was noticed and a rather high expression was observed in skeletal muscle, brain, heart and skin (Fig. 3).

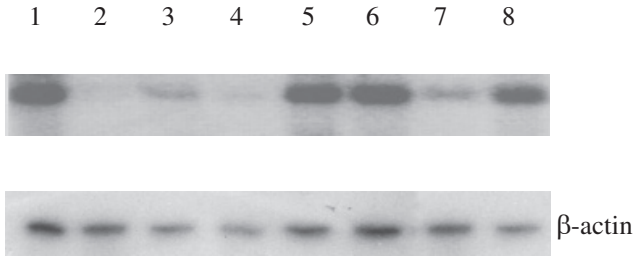

Fig. 3
Expression of the HSRG1 mRNA in different human tissues

Upper row: Northern blot analysis using a probe within the HSRG1 ORF. mRNA samples on the blot: brain (lane 1), pancreas (lane 2), lungs (lane 3), kidney (lane 4), skeletal muscle (lane 5), heart (lane 6), liver (lane 7), and skin (lane 8). Lower row: the same blot hybridized with β-actin cDNA as probe.

Expression and purification of HSRG1 protein

HSRG1 protein was expressed in *E. coli* as a fusion protein. M_r values of the fusion protein and of the HSRG1 protein cleaved off from GST-HSRG1 were 87.2 K and 59.2 K, respectively (Fig. 4A). The HSRG1 protein purified by affinity chromatography was used to raise specific antibodies needed for further analysis.

Cellular location of the HSRG1 protein

The location of HSRG1 protein in cell was determined by two ways. Analysis of the expression of the pEGFP-HSRG1 plasmid in KMB-17 cells revealed that the HSRG1-GFP fusion protein was observed mainly in the cytoplasm of transfected cells, while its signaling in the nucleus was poor (Fig. 5). An analysis using the PSORT II software predicted there was a 47.8% possibility that this protein was located in the cytoplasm. The results obtained by these two methods confirmed that the HSRG1 protein is predominantly located in the cytoplasm.

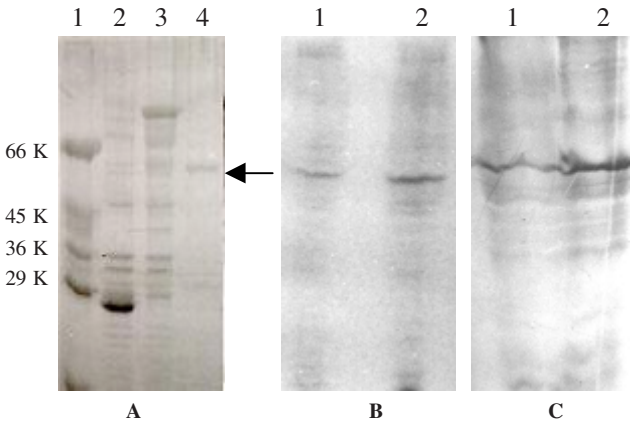

Fig. 4
Expression and purification of HSRG1 protein (A), effect of HSV-1 binding to cells on expression of HSRG1 protein (B), and relationship of expression to phosphorylation of the HSRG1 protein (C)

A. Analysis of purified HSRG1 protein by SDS-PAGE. Protein size marker (lane 1), *E. coli* containing pGEX5x1 plasmid (lane 2), *E. coli* containing pGEX-HSRG1 plasmid (lane 3), purified HSRG1 protein (lane 4).
B, C. Effect of HSV-1 bound to KMB-17 cells on immunoprecipitation of HSRG1 protein.
Cells in [^{35}S]methionine labeling media (B, lane 1), cells with bound HSV-1 in [^{35}S]methionine labeling media (B, lane 2). Cells in [^{32}P]phosphate labeling media (C, lane 1), cells with bound HSV-1 in [^{32}P]phosphate labeling media (C, lane 2).

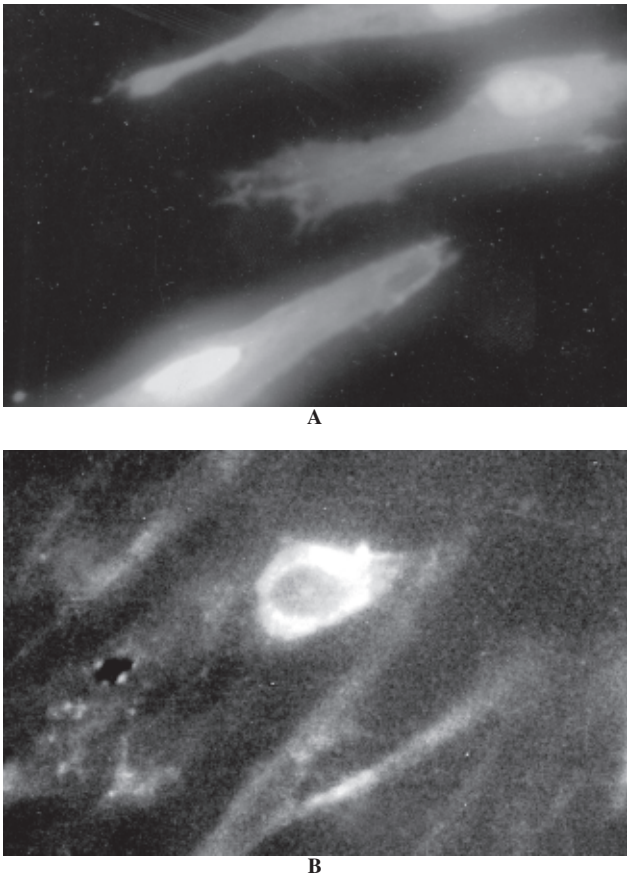

Fig. 5
Subcellular localization of HSRG1 protein in KMB-17 cells

Cells transfected with pEGFPN1 (A). Cells transfected with pEGFP-HSRG1 (B).

Immunoprecipitation

To investigate the relationship between the HSRG1 protein expression in cells and the HSV1 binding to cells, immunoprecipitation was performed with the HSRG1 antiserum and labeled extracts of HSV-1-infected or non-infected KMB-17 cells. The result showed that the HSRG1 protein expression increased in relation to the HSV-1 binding (Fig. 4B). A similar immunoprecipitation experiment with a phosphorylated HSRG1 protein indicated that once the HSRG1 protein was expressed it was phosphorylated in the cell (Fig. 4C).

Discussion

Interactions between virus and cell induce various cellular responses including production of cytokines (Chang and Shaio, 1994) and cellular gene response regulation (Benn *et al*., 1995). In such interactions, the virus binding to specific receptors triggers a signal transduction in cells and induces specific gene responses. Our previous data have indicated that the HSV-1 binding to fibroblasts induces expression of various genes including oncogenes (Li *et al.*, 2000; Li *et al.,* 2002). As one of those genes induced by HSV-1 in fibroblasts, the HSRG1 protein shows an interesting structural homology with the SAND protein family. Immunoprecipitation confirmed that this protein exists in fibroblasts and may change its expression level by HSV-1 stimulation.

As a new protein of unknown function, the HSRG1 protein contains conserved motifs and its homologues represent a family whose members occur in six species. They are characteristic by a conserved domain composed of five PKC phosphorylation sites, six myristoylation sites, one CAMP phosphorylation site, five CK2 phosphorylation sites, and one TYR-phosphorylation site. Although these specific sites have been investigated for their functions, the physiological function of the domain is still not clear. However, the conservative nature of the SAND family implies a potential significance of this family in eukaryotic species. Our initial investigation on their functions in signal transduction revealed that the HSRG1 protein is induced by the HSV-1 binding, it is phosphorylated once it is expressed and is located in the cytoplasm. These clues suggest that the HSRG1 protein probably functions as adaptor in signal transduction in the cells infected with HSV-1. Although, at present, the structural

data about each member of the SAND protein family are only of hypothetical nature, the identification of HSRG1 gene/protein structure and a high level of its expression in human fibroblasts with bound HSV-1 allows us to infer a potential significance of this protein in the cell. The conserved domain of the SAND protein family could be considered a component of signal transduction pathway in different species.

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