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Stress-Activated Kinase Signaling in Breast Cancer

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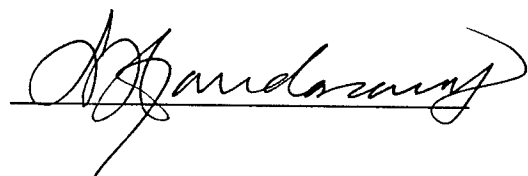
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13. ABSTRACT (<i>Maximum 200 Words</i>) Caspase-mediated cleavage of PAK2 is a hallmark of the apoptotic response accompanied by strong JNK activation. The purpose of this study is to characterize the PAK-JNK signaling pathway and to identify signaling components mediating the JNK activation. In this study we isolated and identified a substrate of p21-activated kinase PAK which might be a downstream regulator in signaling to the Stress-activated kinase module (SAPK) and the morphological response observed in cells undergoing apoptosis. Peptide mass fingerprinting and immunological methods identified this protein as the guanine nucleotide exchange factor GEF-H1/KIAA0651. GEF-H1 is strongly phosphorylated by PAK. The phosphorylation site was identified using deletion mapping, phosphopeptide/-amino acid analysis and in vitro mutagenesis. The central dbl-homology domain of the exchange factor mediates strong JNK activation, which seems not to be mediated by the known SAPK-regulatory GTPases Rac and Cdc42. Using in vitro exchange factor assays we observe activation of RhoA, but not Rac1. Currently, we test the hypothesis of PAK mediating JNK activation via GEF-H1 and RhoGTPases.				
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Table of Contents

Cover.....	
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	12
Appendices.....	13

INTRODUCTION

Cleavage of the protein kinase PAK2 is a hallmark of apoptosis and suggested to mediate activation of the stress-activated protein kinase JNK1 (Rudel et al., 1998). In addition, the morphological response during apoptosis, including cell contraction, membrane blebbing and apoptotic body formation, is inhibited when PAK signaling is blocked using dominant negative PAK1 (Rudel and Bokoch, 1997). PAK kinase is a well characterized effector of the small GTPases Rac and Cdc42 and an important regulator of the actin cytoskeleton. Cytoskeletal alterations might therefore be involved in the pathway activated by caspase-dependent activation of PAK2.

We designed an experimental approach to characterize the signaling pathway controlled by p21-activated kinase and to identify immediate downstream regulators involved in this response.

BODY

As stated in my last annual report we performed a biochemical two-step screen to identify substrates of p21-activated kinase [technical objective 3]. One strongly interacting and phosphorylatable protein was identified as the guanine nucleotide exchange factor GEF-H1/KIAA0651.

As suggested by the database sequence submissions most of the differences in the nucleotide sequences between KIAA0651 and GEF-H1 might be due to sequencing errors. We received cDNA clones for KIAA0651 (Ishikawa et al., 1998) and GEF-H1 (Ren, et al., 1998) and completely sequenced both cDNAs. Indeed, DNA sequencing revealed that the published GEF-H1 cDNA sequence contained many errors including a frameshift. The corrected GEF-H1 sequence extends for an additional 300 nucleotides until a stop codon is reached. In summary, re-evaluation of the DNA sequence revealed that KIAA0651 is almost identical to GEF-H1 except an amino-terminal region (Fig. 2).

The recently deposited human genome sequence revealed that the remaining differences might be due to alternative splicing of the GEF-H1/KIAA0651 pre-mRNA since exons for both isoforms are present in a sequential array (Fig.1). Notably, the initially published but later corrected sequence submission for KIAA0651, might also arise by alternative splicing connecting exons 2-3-5 instead of 2-3-4-5. The KIAA0651 cDNA clone we received and used in our experiments does not contain exon 4.

The corrected GEF-H1 cDNA sequence encodes a 986 aa protein containing several recognizable motifs. An amino-terminal zinc finger (amino acid 40-86), central dbl- (aa 242-437) and pleckstrin homology (aa 472-571) domains typical for RhoGTPase exchange factors, and a coiled-coil region (aa 798-869, see also Fig.2). The extreme carboxy-terminal region showed homology to nucleolin C23 and neurofilament triplet M protein (aa 900-980). The KIAA0651 cDNA clone we obtained encodes a 912 aa protein which contains all of the above motifs but the zinc finger.

Several attempts to isolate the cDNA for the KIAA0651-2 (see Fig.1) by reverse transcription of RNA and subsequent polymerase chain reaction failed. As sources for RNA we used HeLa and Jurkat cells. From these cells only the short form KIAA0651-1 was obtained.

As published by Ren et al. (1998), GEF-H1 is a microtubule-localized guanine nucleotide exchange factor. Recombinant GEF-H1 was shown to possess exchange factor activity toward RhoA,-B,-C and Rac1 but not Ras, Cdc42 or TC10. Microtubule localization required

determinants carboxy-terminal from the DH/PH exchange factor domain. However, it has not been shown that this region is sufficient for localization.

We constructed several HA (hemagglutinin), eGFP-(enhanced green fluorescent protein) and GST (glutathione S-transferase) fusion constructs for the following functional analysis. The regions used for these purposes are presented in Figure 2.

Binding experiments showed that both KIAA0651 and GEF-H1 interact with the PAK kinase domain fused to glutathione S-transferase (GST-PAK1). Using the separately expressed regions of GEF-H1/KIAA0651 we could show that the interaction with GST-PAK was mediated by the carboxy-terminal region (aa 572-986). A carboxy-terminally truncated GEF-H1 (GEF-H1 Δ 896: aa 1-895) was not pulled down with GST-PAK1 confining the PAK interacting region to amino acids 896-986 (data not shown).

GST-PAK1 specifically phosphorylated GEF-H1/KIAA0651 in the carboxy-terminal region (aa 572-986). Phosphorylation was greatly diminished when the deletion extended to amino acid 895 (Fig.3A). On the other hand, a GST-fusion protein comprising amino acids 887-986 was not phosphorylated. In tryptic phosphopeptide maps from phosphorylated GST-CC (data not shown) only one phosphorylated spot was observed. Phosphoamino acid analysis unambiguously determined serine as the phosphorylated amino acid. In the suggestive region the serine at position 886 (RRRS⁸⁸⁶) represented a candidate phosphorylation site for PAK. We mutated this serine to alanine and analyzed phosphorylation in *in vitro* kinase assays. As shown in Fig.3B the mutated GST-CC (aa 572-986, S886A) is not phosphorylated suggesting that serine 886 is the PAK phosphorylation site.

To visualize endogenous GEF-H1/KIAA0651 protein, we generated a polyclonal rabbit antiserum. Glutathione S-Transferase was fused to amino acids 697-779 of GEF-H1 and used for immunization of two rabbits. The polyclonal antiserum can detect GEF-H1 in Jurkat lymphocytes and various breast cancer cell lines (Fig.4). It is notable that only one specific protein is detected in Jurkat T cells and the breast cancer cell lines. The size of this protein corresponds to the predicted size of GEF-H1 (111.5 kDa). Expression in the three tested breast cancer cell lines is heterogenous with MDA231 having high and SKBR-3 having almost undetectable amounts of GEF-H1/KIAA0651.

Next, we analyzed whether GEF-H1 was capable to mediate exchange of guanine nucleotides *in vitro* using recombinant GTPases. For this analysis we immunoprecipitated GEF versions from transfected Cos-1 cells. As shown in Fig.5 GEF-H1 and KIAA0651 both exhibited strong exchange activity toward RhoA. However, we have not been able to detect activity toward Rac1, which is in contrast to the previously published data for GEF-H1 (Ren et al.,

1998). RhoA exchange activity can be detected in GEF-H1 carboxy-terminally truncated versions ($\Delta 896$, $\Delta 574$, see Fig.5 and data not shown). In addition, we could detect GEF-activity in GST-PAK pulldown fractions and immunoprecipitated endogenous GEF-H1 from Jurkat lysates. All GEF proteins tested only exhibited Rho, but not Rac exchange activity. Interestingly, the isolated DH/PH domain (238-571) expressed in Cos-1 cells did not have any measurable exchange factor activity.

To complement the *in vitro* exchange assays we analyzed the Rac- and Cdc42 activity status in cells. A method to trap activated GTPases using the Rac-/Cdc42 binding domain of PAK (PBD) has been developed in our laboratory (Benard et al., 1998). Cos-1 cells were co-transfected with GEF-constructs and epitope-tagged Rac1 or Cdc42. Activated GTPases were precipitated using a GST-PBD (Glutathione S-transferase-PAK binding domain) protein attached to glutathione agarose beads and visualized by western blotting. All GEF-constructs (see Fig.2) except the positive control (oncogenic db1) did not show activation of Rac1 or Cdc42 (data not shown).

Kinase activity of PAK can also serve as a measure for the activity of GTPases. Full length PAK1 was co-transfected with the DH/PH domain of GEF-H1/KIAA0651. The kinase activity of immunoprecipitated PAK was analyzed with myelin basic protein as a substrate. We did not detect activation of PAK kinase (data not shown), again indicating that Rac or Cdc42 were not activated by GEF-H1.

The SAPK JNK can be strongly activated by Rac and Cdc42 (Minden et al., 1995), however, Rho has been demonstrated to mediate JNK activation, too (Alberts, 1998). Therefore, JNK is another indicator for activated GTPases in the cells. In light of my objectives, it was important to investigate whether GEF-H1 is capable of activating this pathway since this factor might represent a link between PAK and the SAPK module. Gef-version (full length, $\Delta 896$, $\Delta 574$, see Fig.2) and KIAA0651 did not significantly activate co-transfected JNK1 (Fig.6). The isolated DH/PH domain (238-571) strongly activated JNK (Fig.6 A,B), whereas the amino-(1-237) and carboxy-terminal region (572-986) did not (data not shown). As seen in Fig.6B the DH domain was sufficient to mediate JNK1 activation. Increasing amounts of transfected DH/PH also increased activation of JNK (data not shown). These data demonstrate that the exchange factor domain of GEF-H1/KIAA0651 is functional in mediating JNK activation but inactive in the context of the full length molecules. Since GEF-H1 $\Delta 574$, which is missing the region carboxy-terminal of the DH/PH domain, is inactive in the JNK assay this indicated that the amino-terminal region might downregulate exchange activity.

The task to analyze JNK activation by PAK2 in co-transfection experiments [technical objective 2, task 2 & 3] could not be accomplished due to a recently discovered problem with our PAK2 constructs. Propagation of the plasmids in E.coli led to several mutations in these constructs rendering the kinase inactive. The extent of mutations in these plasmids is currently under investigation. New constructs have to be prepared and analyzed for expression and kinase activity.

In initial microscopical studies in HeLa and Cos-1 cells we could demonstrate that GEF-H1 localizes to the microtubule network as has been shown by Ren et al. (1998). We have been able to show this by indirect immunofluorescence using epitope-tagged GEF-H1 and fluorescence in live cells using eGFP fusion constructs. Deletion of amino acids 896-986 does not influence localization, however, further deletion up to amino acids 572 abolished microtubule localization. Interestingly, KIAA0651 does not seem to localize to microtubules. We conclude from this that the amino-terminal region containing the zinc finger is important for the localization of the exchange factor in addition to determinants present in the carboxy-terminus.

We have been able to assign a PAK-interacting region and PAK phosphorylation site to GEF-H1/KIAA0651 in addition to analyzing GTPase activation by various means. *In vitro* GEF-H1/KIAA0651 showed exchange activity toward RhoA but not Rac1. The isolated DH/PH domain did not have *in vitro* exchange activity. On the other hand, the isolated DH/PH domain strongly activated JNK which seemed not to be mediated by Rac or Cdc42. All GEF versions containing the amino-terminal region do not activate JNK in contrast to the *in vitro* exchange activity data.

We currently do not understand why the DH/PH domain (aa 238-571) of GEF-H1/KIAA0651 is not active in the established *in vitro* exchange assay. One explanation is that activation of RhoA requires determinants in the amino-terminal region and activation of JNK does not, instead, it seemed to be inhibited by this region. This might indicate that another GTPase, which is not Rac or Cdc42, is activating the JNK signaling pathway. Further studies, however, have to be performed to challenge this hypothesis.

Several questions need to be addressed in future studies. Does phosphorylation and/or interaction with PAK influence exchange activity and consequently JNK activation? Is JNK activation in the cell mediated by Rho as suggested by the *in vitro* exchange assays? Does localization influence GEF-activity? Currently, we are developing experimental approaches to answer these questions.

KEY RESEARCH ACCOMPLISHMENTS

- **Identification of GEF-H1/KIAA0651 as a substrate of PAK kinases**
- **Correction of sequencing errors in GEF-H1**
- **Assignment of a PAK binding region in GEF-H1/KIAA0651**
- **Identification of the phosphorylation site in GEF-H1/KIAA0651**
- **Preparation of GEF-H1/KIAA0651 antiserum**
- **Measurement of exchange factor activity and determination of specificity**
- **Activation of JNK by the DH/PH exchange factor domain of GEF-H1/KIAA0651**

REPORTABLE OUTCOMES

King, C.C., **Zenke, F.T.**, Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A., Knaus, U.G., Bokoch, G.M. Sphingosine-stimulated 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates p21-activated kinase (PAK1). J Biol Chem 2000 Jun 16;275(24):18108-13

Zenke, F.T. and Bokoch, G.M. The role of caspase-mediated PAK2 activation. Keystone Symposium in Breckenridge, Colorado on programmed cell death, 4/6/99 to 4/11/99

Zenke, F.T., King, C.C. and Bokoch, G.M. Regulation of PAK activity by autoinhibition and phosphorylation. San Diego Cell Biology Meeting, 5/20/99.

King, C.C., Gardiner, E.M., **Zenke, F.T.**, Bohl, B.P., Newton, A.C., Hemmings B.A., Bokoch, G.M. p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1). J Biol Chem 2000 Dec 29;275(52):41201-9

Zenke, F.T. Function of PAK Kinase activity in Apoptosis. Posterpresentation DOD BCRC Meeting, Atlanta, June 8-11, 2000

CONCLUSIONS

The Guanine nucleotide exchange factor GEF-H1/KIAA0651 has been identified as a novel substrate of PAK kinases. Most importantly, the isolated GEF domain can activate the JNK signaling pathway presumably not via Rac- or Cdc42 activation. The PAK phosphorylation site was mapped in GEF-H1 and its functional relevance for JNK activity is under investigation. It is of interest to understand the mechanism of exchange factor regulation especially since the full length proteins seem to be inactive. In this context, it is also of importance to evaluate if the microtubule-binding status influences its activity. Drugs interfering with the microtubule polymerization status might impact the activity of the molecule and be another means to signal to the MAPK module.

REFERENCES

- Alberts AS, Treisman R. Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1. *EMBO J* 1998 Jul 15;17(14):4075-85
- Altschul, SF, Gish, W, Miller, W, Myers, EW and Lipman, DJ. Basic local alignment search tool. *J. Mol. Biol.* 1990; 215:403-410.
- Benard V, Bohl BP, Bokoch GM Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J Biol Chem* 1999 May 7;274(19):13198-204
- Ishikawa K, Nagase T, Suyama M, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O. Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. *DNA Res* 1998 Jun 30; 5(3):169-176
- Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 1995 Jun 30;81(7):1147-57
- Ren Y, Li R, Zheng Y, Busch H. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J Biol Chem* 1998 Dec 25; 273(52):34954-34960
- Rudel T, Bokoch GM. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* 1997 Jun 6; 276(5318):1571-1574
- Rudel T, Zenke FT, Chuang TH, Bokoch GM. p21-activated kinase (PAK) is required for Fas-induced JNK activation in Jurkat cells. *J Immunol* 1998 Jan 1; 160(1):7-11

Genomic Locus of GEF/H1/KIAA0651

GI 11427616:

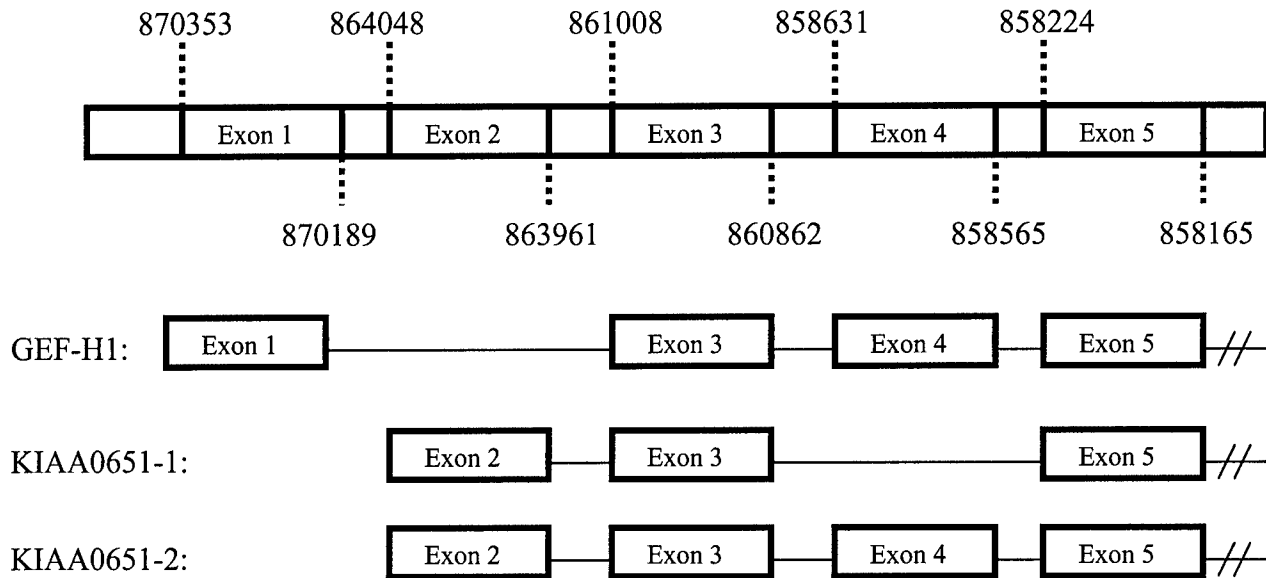


Figure 1: Schematic representation of the genomic locus of GEF-H1/KIAA0651 as deduced from the working draft sequence of the GI 11427616 contig derived from human chromosome 1. The exons were identified by sequence comparison with the cDNA sequence of GEF-H1/KIAA0651 using the BLAST algorithm (Altschul et al., 1990). The numbers give the positions of the exon borders in the contig. The array of the first five exons of the genomic GEF-H1/KIAA0651 locus and their presence in the cDNA splice variants is shown. Note, that the 5'-start site of all mRNA variants have not been confirmed yet.

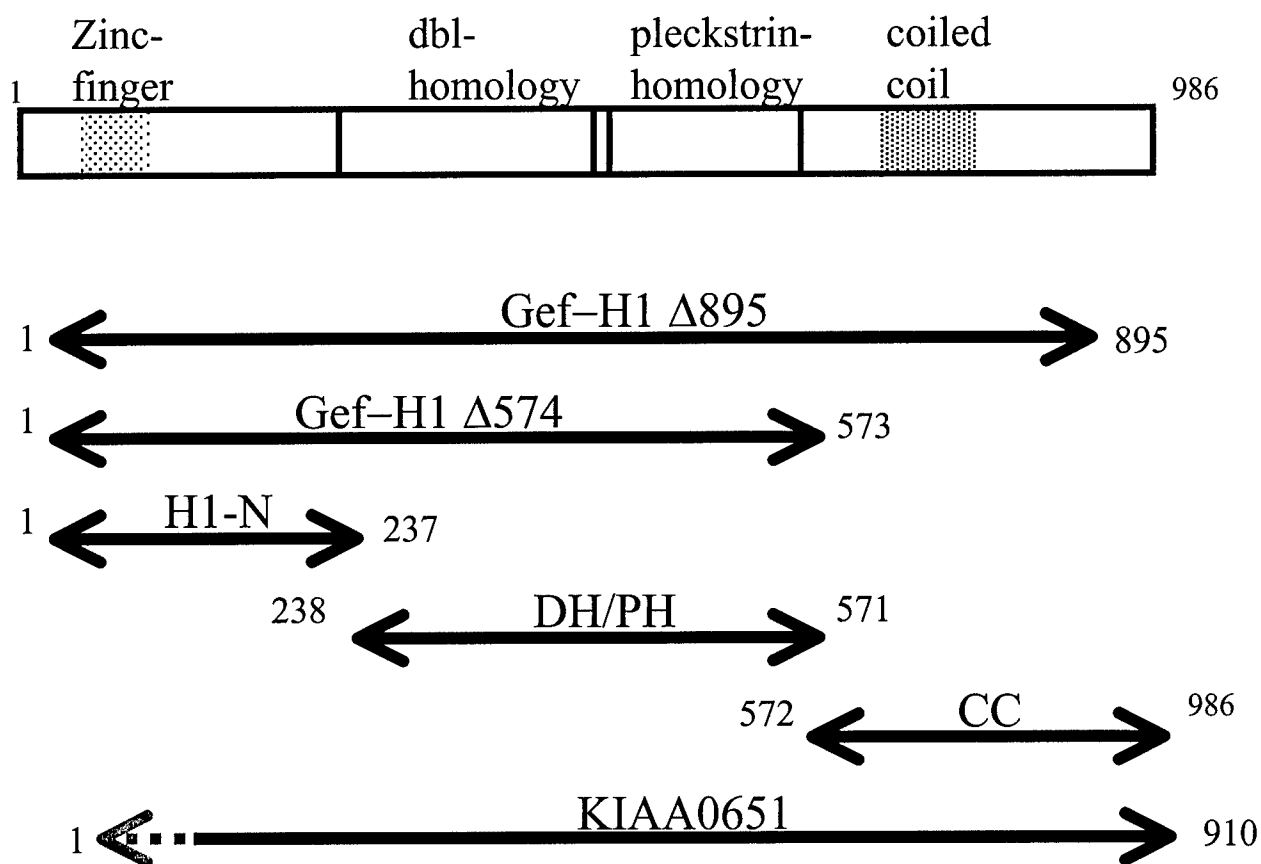


Figure 2: Schematic representation of GEF-H1/KIAA0651 constructs used in this study. The GEF-H1 full length construct is shown at the top with the recognizable motifs indicated. The KIAA0651 isoform we obtained is identical to Gef-H1 except that the amino-terminal region is different and the zinc finger is missing (grey, dotted region). The above regions were inserted into mammalian and bacterial expression plasmids and used to characterize GEF-activity, JNK activation and interaction/ phosphorylation by PAK as mentioned in the text.

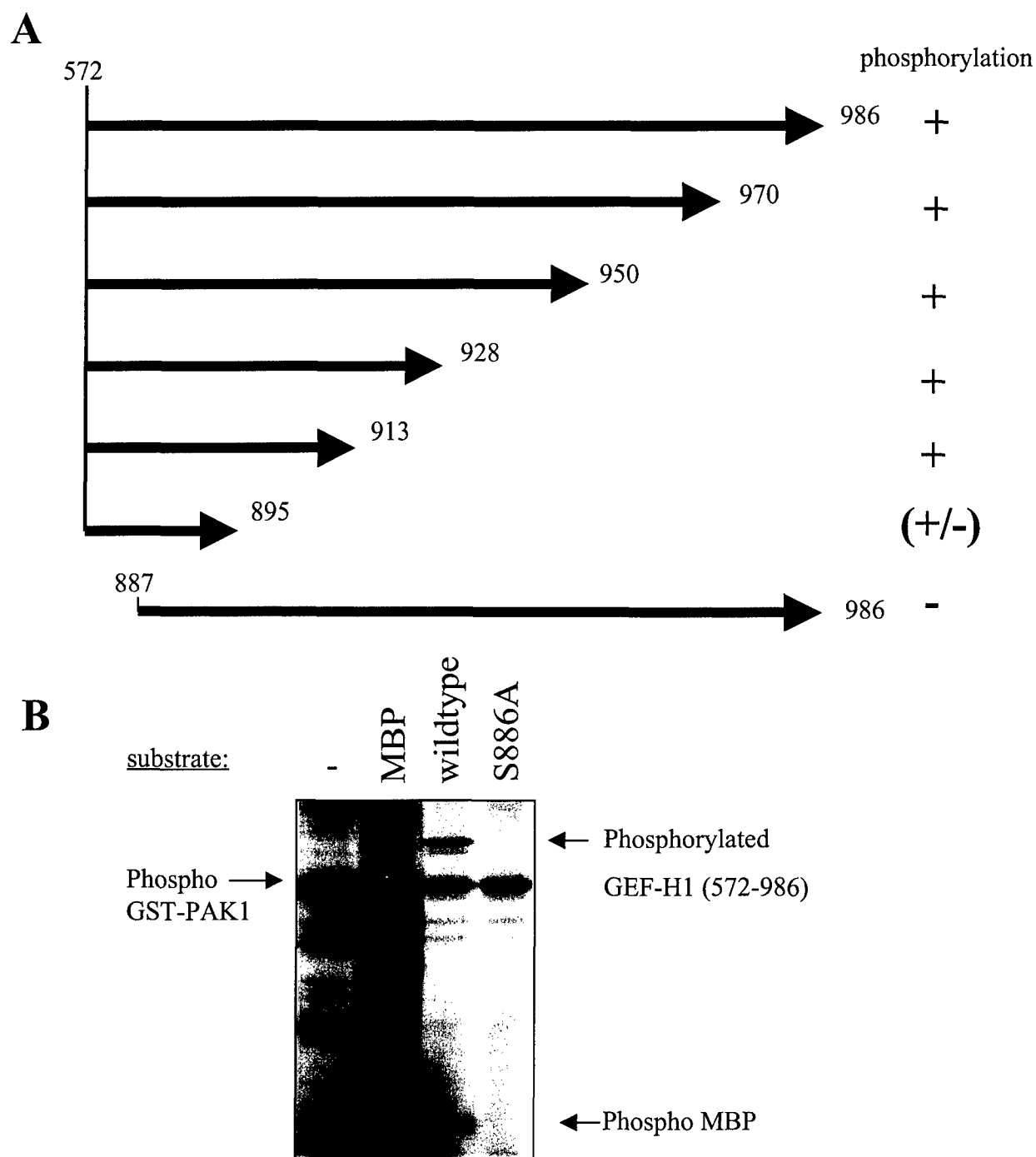


Figure 3: Mapping of the GEF-H1 phosphorylation site. A) Glutathione S-transferase fusion proteins containing the indicated portions of GEF-H1 were tested in *in vitro* kinase assays using GST-Pak1 as a kinase. The efficiency of phosphorylation is indicated on the right (+: phosphorylated; +/-: weakly phosphorylated; -: not phosphorylated). B) *In vitro* kinase assay using the GEF-H1 carboxy-terminus (amino acids 572-986) and the corresponding point mutation (S886A, serine to alanine) as substrates. Myelin basic protein is used as a control substrate in lane 2. GST-PAK1 was present in all reactions. The autoradiograph shown is overexposed to demonstrate that no phosphorylated band is present in the serine to alanine mutated variant. The amount of substrate used in lane 3 and 4 is the same as judged by Coomassie staining.

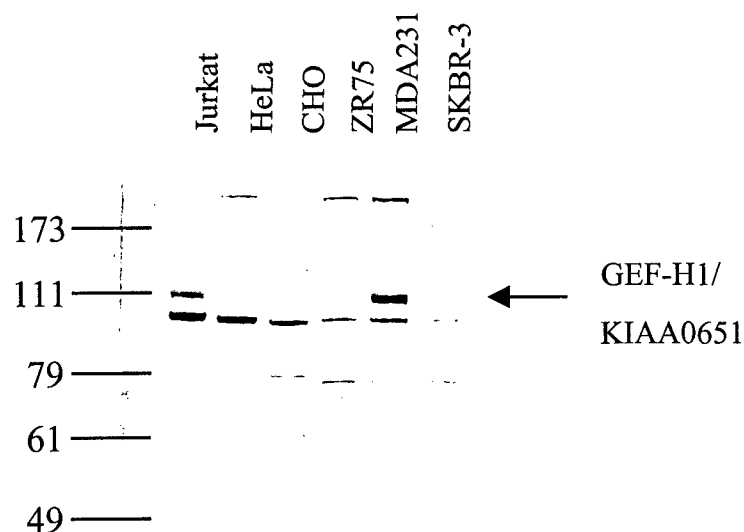


Figure 4: Expression of GEF-H1/KIAA0651 in various cell lines. About 50 μ g of total lysate was electrophoresed on a 10% SDS polyacrylamide gel and transferred to nitrocellulose. The GEF-protein was visualized using the prepared rabbit polyclonal antiserum and an alkaline phosphatase-coupled secondary antibody. The GEF protein was not detectable in HeLa cells but weakly detectable in SKBR-3. Jurkat T lymphoblast cells and MDA231 show equivalent high amounts in the lysates. Jurkat: T-lymphoblast cell line; HeLa: human cervical carcinoma; CHO: chinese hamster ovary; ZR75, MDA231, SKBR-3: human breast cancer cell lines

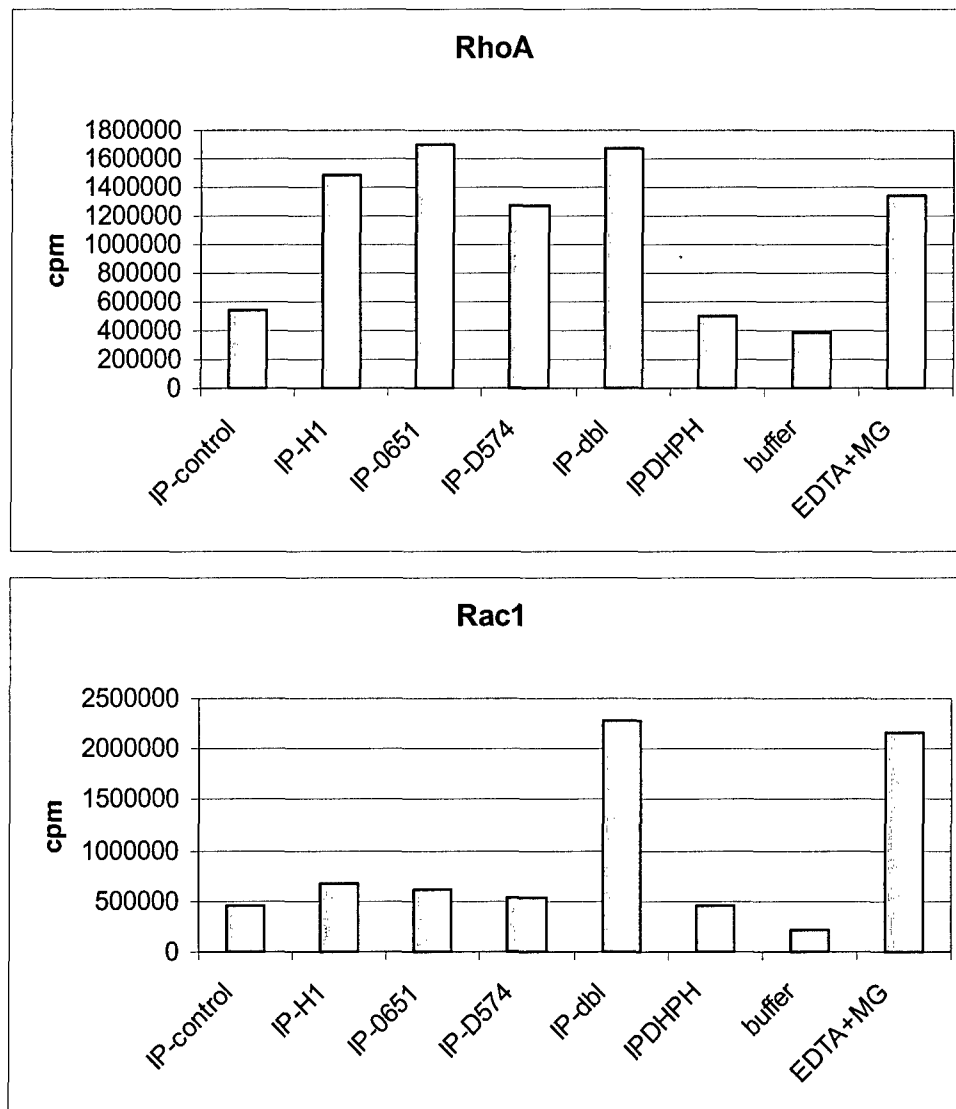
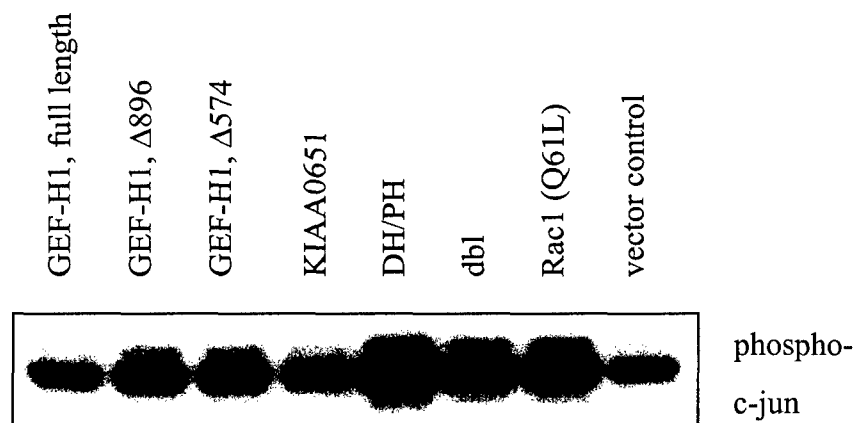


Figure 5: In vitro exchange assay using recombinant RhoA and Rac1. Exchange activity toward RhoA is shown in the upper panel, toward Rac1 in the lower panel. The indicated GEF-proteins (GEF-H1 full length: IP-H1; KIAA0651: IP-0651; GEF-H1, Δ 574: IP-D574; db1 oncoprotein: IP-dbl; DHPH domain of GEF-H1: IP-DHPH) were immunoprecipitated from transfected Cos-1 cells with α -HA antibody, washed extensively and equilibrated in exchange buffer. The recombinant GTPase was added in the presence of radiolabeled GTP γ S[35S] to the protein G-bound exchange factor and incubated for 15 minutes at 30°C. The reactions were stopped with ice cold Stop-buffer and filtered through nitrocellulose filters. The filter-bound radioactivity representing the exchanged guanine nucleotide was counted by liquid scintillation counting. As controls, exchange activity was measured in the presence of immunoprecipitate from Cos-1 control lysates (IP-control) and exchange buffer (buffer). In addition, loading was enforced by EDTA-destabilization in the presence of the radiolabeled nucleotide and subsequent addition of magnesium ions (EDTA+Mg).

A



B

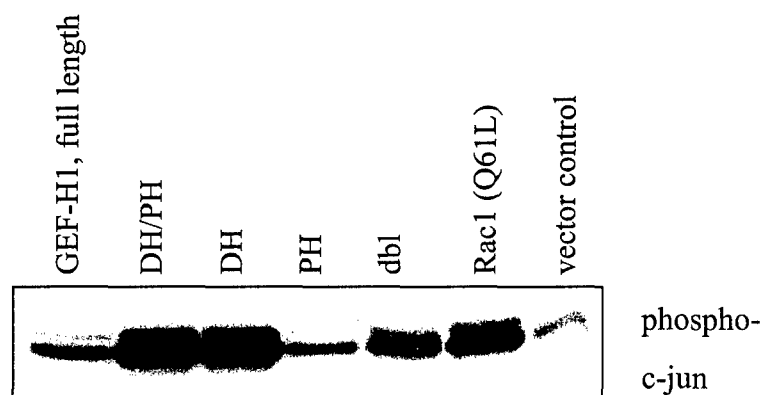


Figure 6: JNK activation assays. Cos-1 cells were co-transfected with the indicated constructs and Flag-tagged JNK1. 24 hours post transfection cells were starved in serum-free medium for another 24 hrs. JNK1 was immunoprecipitated from cell lysates and activity was measured in in vitro kinase assays using GST-c-jun as a substrate. A) All GEF-constructs except the isolated DH/PH domain (aa 238-571) did not significantly activate JNK1. Dbl and activated Rac1 (Q61L) were used as positive controls, vector-transfected cells served as a negative control. B) The DH domain is sufficient to activate JNK1. Controls are the same as in A.



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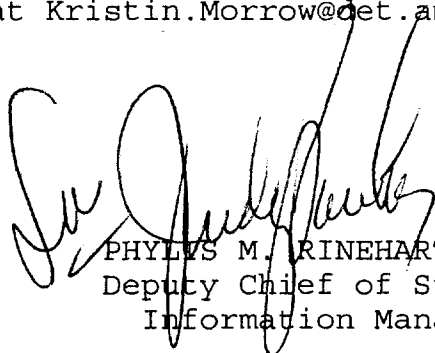
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