Abstract. In a human cancer cell line, we previously found a mutation in codon 322 of the extracellular signal-regulated kinase (ERK2 E322K), the protein showed a faster migration when compared to wild-type in SDS-PAGE and constitutive phosphorylation. However, the reason for the faster migration, and the biochemical and biological properties of the mutation is unknown. In this study, we report that the amino acid charge-change mutation in the common docking (CD) domain is important for fast migration. In vitro binding of ERK2 E322K to MKP1 and RSK2 was lost, resulting in constitutive activation and possibly contributing to a more efficient colony formation in soft agar. We established transgenic flies by carrying the corresponding CD domain mutation, DERK E335K, which developed smaller and rougher eyes compared with the wild-type. Taken together, these data are consistent with ERK2E322K loss of contact with downstream effectors and its constitutive activation, presenting an oncogenic potential and weak abnormality in differentiation.

Introduction

The extracellular signal-regulated kinase (ERK) belongs to the mitogen-activated protein kinase (MAPK) family, which plays important roles in cell growth, differentiation, and survival (1,2). ERK is activated by extracellular growth factors through RTKs (3). ERK is up-regulated with the upstream dual kinase, MEK (4), which phosphorylates threonine and tyrosine residues of the TEY sequence in ERK (5). In contrast, ERK is down-regulated with ERK-regulated phosphatases with dual-specificity phosphatase activity, keeping ERK activation under control (2). Activated ERK activates RSK in the cytoplasm, enters the nucleus and phosphorylates various transcriptional factors such as ELK-1 (6) through phosphorylation at serine/threonine residues of the target proteins (7). It has been reported that various signal transductions are mediated through the acidic amino-acid rich common docking (CD) domain. A gain-of-function mutant within this region was first reported in sevenmaker of Drosophila (DERK D334N/sem) (8), resulting in loss of binding to MKP1 (9,10) and RSK (11). We previously reported a CD domain mutant, ERK2 E322K, from a human cancer cell line that was detected as a faster-migrating band in SDS-PAGE (12). Herein, we examined the biochemical and biological properties of the faster-migrating band in SDS-PAGE (12). Herein, we examined the biochemical and biological properties of the faster-migrating ERK2 E322K and found that other amino acid charge-change mutations at other sites in the CD domain, including human counterparts of ERK sem/D321N, resulted in faster migration. ERK2 E322K induced the anchorage-independent growth of Balb3T3 cells in soft agar and weak abnormality in Drosophila eye development.

Materials and methods

Cell lines and culture. HaCaT (13), HEK 293T, HSC6, Ca922, Saos-2 and Balb3T3 cells were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceuticals, Tokyo, Japan) and supplemented with 10% FBS (JRH Biosciences, Tokyo, Japan) and humidified 5% CO₂/95% air.

Construction of HA-tagged and LTR plasmids. ERK2 or ERK2 E322K, originally cloned in pGEM®-T easy vector (Promega, Madison, WI), were PCR-amplified using 5’-TGATATCTTATGGCGGCGGCGG-3’ (forward) and 5’-GGCTCGAGTATCTTATGGCGGCGGCGGCGGCGG-3’ (reverse) primers,
digested with EcoRV and XhoI and cloned in-frame in pcDNA3-2HA. Two tandem copies of hemeagglutinin epitope sequences were inserted at HindIII and EcoRI sites.

To express in mouse cells the NorI fragment of ERK2, originally cloned in pGEM-T easy vector, the cells were cloned in a pLTR-SA vector (14) where the BamHI site was changed to NorI site by blunting the BamHI site followed by NorI linker ligation. A neomycin derivative (G418) gene was inserted.

Plasmid purification was performed using an endofree DNA purification kit (Qiagen Japan, Tokyo).

Site-directed mutagenesis. In vitro mutagenesis of ERK2 was performed as described in the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla) using the primer sets: D321K/5'-ATTACGACCCGAGTAAAGAGCCCATC-3' (forward) and 5'-GATGGGCTTTTACTCGGGTGTAAT-3' (reverse), K321N/5'-CGACCCGAGTAACGAGCCCATC CG-3' (forward) and 5'-CGGCGATGGGCTCGTTACTCGGGTCG-3' (reverse), D318K/5'-CTGGAGCAGTATGGAACCGAGT-3' (forward) and 5'-ACTCGGTTTGTATTGCTCCAG-3' (reverse), E250K/5'-TGGATCCCCATCAAAGTTGGAATTG-3' (forward) and 5'-CAATTCAGGTCTTTTGTGATGGGGATCCA-3' (reverse), and M13L/5'-GCGGGCCCGGAGCTGGTCCGCGGGCAGG-3' (forward) and 3'-CCTGCCCGCGGACCAGCTCCGGGCCGC-3' (reverse). The mutations were introduced in HA-ERK2wt. D321N was constructed from D321K. The mutant constructs were confirmed by sequencing (ABI PRISM version 1, ABI Japan, Tokyo).

Treatment with EGF and mammalian cell lysate preparation. Ca922 cells were serum-starved in DMEM for two days, and stimulated with EGF (10 ng/ml, Sigma-Aldrich, MO) for 1 h. Cells were then washed with ice-cold PBS and lysed in NP-40 lysis-buffer as previously described (12). Nuclear extracts were prepared from Saos-2 cells as previously described (15).

Western blotting. Western blotting was performed as previously described (16). Anti-MKP1, anti-ERK2 (D-2), anti-ERK1 (C-16) and anti-RSK2 (C-19) (Santa Cruz, CA), as well as anti-HA (Boehringer Mannheim, Germany) and the HRP conjugates (New England Biolabs) were used.

GST-tagged vector, bacterial growth and lysate preparation, and pulldown assay. GST-ERK2wt and GST-ERK2E322K were constructed (17), and used to transform E. coli BL21 (DE3), which was induced and lysed as previously described (16). GST-ERK2wt, GST-ERK2 E322K and GST-vector containing bacterial lysates were used for the pulldown assay (18) of MKP1 and RSK2 proteins in Ca922 total cell and Saos-2 nuclear lysates.

Colony formation assay in soft agar. Balb3T3 cells were lipofected (LF2000, Invitrogen) by using the LTR-ERK2wt or -ERK2E322K or -vector alone according to the manufacturer’s directions, and grown with 400 μg/ml G418. For clones stably expressing the wild-type or mutant proteins, the cells were grown for 2 days in the presence of G418 and used for the soft agar assay. A bottom agar in DMEM with 0.5% agarose and top agar with 3000 cells and 400 μg/ml G418 in DMEM and 0.33% agarose were made, incubated in 5% CO2 and monitored for colony formation as previously described (17), with a few modifications.

Preparation of Drosophila cDNA. Total RNA was isolated using a TRizol® reagent (Invitrogen) and cDNA was prepared with an AgPath-ID™ one-step RT-PCR kit (ABI) as described in the respective kits.
**pUAST-DERK** plasmid construction. pUAST (19) is a P-element vector (pCaSpeR3) containing the P3' and P5' ends of P element and the white gene which acts as a selection marker for successful integration into the *Drosophila* genome. To construct the pUAST-DERKwt and pUAST-DERK E335K plasmids, *Drosophila* ERK cDNA was amplified by using 5'-TAGCGGCCGCATGGAGGAATTTAATTCG-3' (forward, with NotI site) and 5'-AACTCGAGTTAAGGCGCATTGTCTGG-3' (reverse, with XhoI site) primers, and amplicon was inserted into pGEM-T easy vector. The clones were PCR-amplified using the above-mentioned forward and 5'-AAGTCGACTTAAGGCGCATTGTCTGG-3' (reverse, with SalI site) primers and cloned into the pUAST vector. Then, *in vitro* mutagenesis was performed using 5'-GATCCTGGAGATAACTGGCTGTCGCTGAAG-3' (forward) and 5'-CTTCAGCGACAGGCTTATCTCCAGGATC-3' (reverse) primers and the mutagenized sequence was confirmed by sequencing.

**Establishment of transgenic flies.** The plasmids pUAST-DERKwt or -DERKE335K or the empty vector were injected in fertilized *Drosophila* eggs and the P-element-mediated germ line transformation was carried out as described earlier (20). F1 transformants were selected on the basis of white eye-color rescue (21). The established transgenic flies were crossed with the transgenic fly line (number 16) carrying GMR-GAL4 (22).

**Scanning electron microscopy.** Adult transgenic flies were anesthetized, mounted on stages and observed with a VE-7800 scanning electron microscope (Keyence Inc., Osaka, Japan) in a high vacuum mode.

**Statistical analysis.** A comparison between the numbers of colonies formed by wild-type or mutant ERKs in soft agar was made with unpaired Student’s t-test for continuous outcomes. P-values were two-sided and p<0.05 was considered statistically significant.

**Results**

ERK2 proteins with amino acid charge-change mutations in the CD domain migrate faster in SDS-PAGE. Our earlier study revealed that the faster migrating mutant ERK2 E322K, found in the HSC6 cancer cell line (12), was ~40 kDa. Since the protein was detected with a C-terminal anti-ERK2-specific antibody, we excluded the presence of premature termination as the reason for faster migration. A possibility is the presence of an internal start site at codon 13, resulting in a constructed HA-ERK2 M13L, which was examined for migration, while another possibility is the amino acid charge-change mutation in the CD domain or in the MAPK insert region. Thus, we constructed other HA-tagged mutants in the CD domain (23) and MAPK insert region (24) as shown in Fig. 1A.

293T cells transiently expressing HA-ERK2 mutants showed that charge-change mutation in the MAPK insert region (ERK2 M13L) did not markedly affect its migration in SDS-PAGE. However, mutants in the CD domain (ERK2 E322K, ERK2 D321N and ERK2 D318K) resulted in a faster migration (Fig. 1B). In addition, the double mutant (ERK2 E322K + D318K) migrated as ERK2 E322K but not as ERK2 E322K (Fig. 1C), confirming that the amino acid charge-change mutation in the CD domain resulted in faster migration, whereas the mutation in the MAPK insert region did not. Not surprisingly, the ERK2 M13L mutant did not migrate as ERK2 E322K but the ERK2 M13L + E322K double mutant did (Fig. 1D). It is likely that any other charge-change mutations in the CD domain of ERK2 may also result in faster migration.

ERK2 E322K is constitutively phosphorylated and loses its interaction (*in vitro*) with MKP1 and RSK2. The constitutive phosphorylation of ERK2 E322K in HaCaT and HSC6 cells. Cell lysates were prepared from EGF-treated HaCaT and HSC6 cells for the indicated time period. The blotted lysates were then probed using anti-phospho-tyrosine and anti-ERK2 antibodies. (B) Loss of binding of ERK2 E322K to MKP1. Cell lysates were prepared from serum-starved or EGF-treated Ca922 to induce MKP1. After pulldown with GST-ERK2 wt or GST-ERK2 E322K, the amounts of MKP1 were examined by Western blotting using anti-MKP1 antibody. (C) Loss of binding to RSK2 by pulldown assay. The binding of RSK2 to GST-ERK2 wt or GST-ERK2 E322K were examined as described in Fig. 2A, using anti-RSK2 antibody.

**Figure 2. Biochemical and biological characteristics of ERK2 E322K mutation.** (A) Constitutive phosphorylation of ERK2 E322K in HaCaT and HSC6 cells. Cell lysates were prepared from EGF-treated HaCaT and HSC6 cells for the indicated time period. The blotted lysates were then probed using anti-phospho-tyrosine and anti-ERK2 antibodies. (B) Loss of binding of ERK2 E322K to MKP1. Cell lysates were prepared from serum-starved or EGF-treated Ca922 to induce MKP1. After pulldown with GST-ERK2 wt or GST-ERK2 E322K, the amounts of MKP1 were examined by Western blotting using anti-MKP1 antibody. (C) Loss of binding to RSK2 by pulldown assay. The binding of RSK2 to GST-ERK2 wt or GST-ERK2 E322K were examined as described in Fig. 2A, using anti-RSK2 antibody.
expression of MKP1 was at its peak at 1 h in HSC6 cells (data not shown).

The CD domain mutation of DERKsem was reported to have reduced binding to a number of downstream effectors which included MKP1/2/3, and RSK in vitro and in vivo (9-11). As EGF induces MKP1/2, we used EGF-treated Ca922 cell lysates to examine the interaction of MKP1 with GST-ERK2wt and -ERK2 E322K. The pulldown assay showed that ERK2 wt bound to MKP1 but ERK2 E322K did not (Fig. 2B). The result indicates that this CD domain mutation of ERK2 E322K lost its capacity to bind to MKP1.

In Fig. 2C, it is evident that ERK2 wt strongly binds to RSK2 but not to ERK2 E322K. Thus, as seen in Fig. 2B and C, together they suggest that the ERK2 E322K mutation resulted in loss of communication with the downstream target proteins, MKP1 and RSK2, both of which were reported to contribute to the constitutive activation of ERK2.

Anchorage-independent growth of ERK2 E322K-expressing Balb3T3 cells in soft agar. As we initially found this mutation in a human cancer cell line and it was constitutively activated, we explored whether this mutant ERK2 E322K was able to enhance anchorage-independent growth. Balb3T3 cells stably (Fig. 3A) and transiently (Fig. 3B) expressing ERK2 E322K formed more numbers of colonies than the cells expressing wild-type ERK2 (Fig. 3A, *p=0.0035 and B, *p=0.065).

Effect of CD domain mutation DERK E335K in Drosophila eye development. To delineate the physiological changes induced by the CD domain mutation in Drosophila eye development, we established transgenic Drosophila by carrying the DERKwt or the CD domain mutation DERKE335K, corresponding to the human ERK2 E322K. The transgenic fly was crossed with GMR-GAL4 driver strain to examine the effect of DERKE335K on eye development, and compared with the sevenmaker mutant DERKsem. Scanning electron microscopy of the transgenic fly for DERK E335K showed a more severe abnormality in adult Drosophila eye morphology than the transgenic flies for DERKwt, by exhibiting a perturbed eye development with a smaller size and rougher eyes (Fig. 4A). In Fig. 4B, a comparison of the DERK sem mutant expressing fly and the control fly eye. Resolutions of x200 and x800 are shown as in A.

Discussion

Our earlier report on the CD domain mutation in the human cancer cell line, HSC6, demonstrated its constitutive phosphorylation by MEK and a faster migration in SDS-PAGE (12). Constitutively phosphorylated (25) and overexpressed (26) ERK had been reported in many cancers such as hepatocellular carcinomas, renal cell carcinomas, and gastric adenocarcinomas (27-29). In this study, we found that the other CD domain mutations with amino acid charge-change migrated faster and thus, may be constitutively active. Such a CD domain mutation was first reported as a sevenmaker mutation in Drosophila (8).
ERK2 activity depends on the interaction with upstream and downstream effectors. The CD domain is necessary for interaction with several downstream effectors, and the MAPK insert region is important for interaction with the upstream effector, PEA-15 for nuclear entry (30). MKP1 is a phosphothreonine/tyrosine dual-specificity phosphatase, which dephosphorylates ERK1/2 in *in vitro* and the expression of MKP1 in the cell block activation of ERK1/2 (31). In addition, ERK1/2 phosphorylates and stabilizes the MKP1 protein (32) and in turn, MKP binds to the CD domain and inactivates ERK2 (23). Our preliminary results with control HaCaT cells showed that the time course of MKP1 stimulation resulted in the decrease of ERK1/2 activation. However, this activation was not affected in HSC6 cells (Fig. 2A). Based on these results, further experiments conducted by us show that ERK2E322K mutation lost its binding to MKP1, thus avoiding inactivation. Since it is known that MKP3 interacts with ERK2 through the CD domain, it is presumed that the interaction between them is also lost. It is known that RSK physically interacts with ERK2 and negatively regulates the nuclear entry of ERK2 (11). It was recently reported that mutant ERK2E320K (rat ERK2 homolog to human ERK2E322K) is actively translocated into the nucleus as efficiently as ERK2 wt (30). Unlike the MAPK insert mutant ERK2, the CD domain double mutant D316A and D319A failed to interact to MKP3 and RSK1 in a yeast two-hybrid system showing the importance of the CD domain in the interaction of ERK2 with its cellular targets (33). It is thus suggested that loss of binding to MKP1 and RSK2 conferred to the constitutive activation on ERK2E322K, though more experiments should confirm whether this facilitated proliferation and oncogenic transformation. Our preliminary data showed three important results: i) HSC6 cells maintained phospho-threonine/tyrosine and phosphothreonine/tyrosine ERK2 proteins even at 4 h after EGF stimulation, ii) MKP1 levels were maintained at high levels at 1 h after EGF stimulation and iii) serum-starved HSC6 cells (expressing ERK2E322K, i.e. 75% of the total ERK2 in the cell) had two times more kinase activities than the control HaCaT cells (Arvind et al., unpublished data) showing its constitutive activation by the mutant ERK2.

The constitutively active mutant ERK2E322K is likely to present an oncogenic potential, due to loss of control with negative signaling. It should be noted that this mutation was originally identified in a human cancer cell line. These results are consistent with enhanced anchorage-independent growth by this mutation. We showed that the mutant ERK2E322K overexpressing Balb3T3 cells induced more numbers of colonies and thus, anchorage-independent cell growth. Though the cells transiently expressing ERK2E322K formed more numbers of colonies than those expressing wild-type (Fig. 3B), the values were not statistically significant (p=0.065). However, a higher number of colonies formed by the stable cells expressing ERK2E322K were statistically very significant (*p=0.00355*) (Fig. 3A).

The DERKD335K CD domain mutation that we constructed, which corresponded to the ERK2E322K mutation, revealed that this mutation resulted in a perturbed, smaller and rougher eye development in *Drosophila*. In Fig. 4B, though the external eye development of DERKD334N/sem did not appear to be as abnormal as DERKD335K, DERKD334N/sem induced an R7 differentiation and abnormal wing formation, which were not observed with DERKD335K in our study (data not shown). This may be due to UAS mediating overexpression of ERK2, and inducing marked morphological changes such as small and rough eyes. Since DERKD334N/sem is not overexpressed, it did not show such morphological abnormality. The difference in position of the mutation and the difference in the amino acid charge-change between DERKD335K and DERKD334N/sem may influence the differences in inducing abnormality. It has been reported that the Drosophila ERKD334N/sem mutant failed to bind RSK2 (11). The importance of RSK in PC12 cell differentiation was reported earlier (34).

In summary, our results suggest that the ERK2E322K CD domain mutation is involved in enhanced cell growth and abnormal differentiation. Further studies of the CD domain are needed to elucidate the more novel functions of ERK2.

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