Increased levels of the HER1 adaptor protein Ruk/CIN85 contribute to breast cancer malignancy

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The adaptor protein regulator for ubiquitous kinase/c-Cbl-interacting protein of 85 kDa (Ruk/CIN85) was found to modulate HER1/EGFR signaling and processes like cell adhesion and apoptosis. Although these features imply a role in carcinogenesis, it is so far unknown how and by which molecular mechanisms Ruk/CIN85 could affect a certain tumor phenotype. By analyzing samples from breast cancer patients, we found high levels of Ruk/CIN85 especially in lymph node metastases from patients with invasive breast adenocarcinomas, suggesting that Ruk/CIN85 contributes to malignancy. Expression of Ruk/CIN85 in weakly invasive breast adenocarcinoma cells deficient of Ruk/CIN85 indeed converted them into more malignant cells. In particular, Ruk/CIN85 reduced the growth rate, decreased cell adhesion, enhanced anchorage-independent growth, increased motility in both transwell migration and wound healing assays as well as affected the response to epidermal growth factor. Thereby, Ruk/CIN85 led to a more rapid and prolonged epidermal growth factor-dependent activation of Src, Akt and ERK1/2 and treatment with the Src inhibitor PP2 and the PI3K inhibitor LY294002 abolished the Ruk/CIN85-dependent changes in cell motility. Together, this study indicates that high levels of Ruk/CIN85 contribute to the conversion of breast adenocarcinoma cells into a more malignant phenotype via modulation of the Src/Akt pathway.

Introduction

Worldwide, breast cancer is one of the most common types of tumors found in women. The development of breast cancer is a complex multistage process that involves changes in the expression of multiple genes. Endocrine therapies of breast cancer are designed mostly to limit estrogen biosynthesis and to inhibit estrogen receptor signaling (1). However, de novo resistance to endocrine therapy is the major problem in breast cancer treatment. It is now widely documented that, in addition to estrogen-induced signaling, the inappropriate regulation of growth factor signaling cascades induces development of breast adenocarcinoma and also promotes anthracycline failure in breast cancer cells (1). A large fraction of breast tumors carry oncogenic mutations that cause hyperactivation of the Raf/extracellular signal-regulated kinase (ERK) and/or phosphatidylinositol-3’ kinase (PI3K)/Akt signaling pathways (2,3). In particular, activation of these pathways can be triggered by the epidermal growth factor (EGF) receptors HER1 and HER2/ErbB2, which are upregulated and associated with a poor prognosis in many human breast cancers (3).

The epidermal growth factor receptor (EGFR) signaling and that of other receptor tyrosine kinases involve adaptor/scaffold proteins, which by itself can act either as tumor suppressors or oncogenes (4). The adaptor/scaffold protein regulator for ubiquitin kinase (Ruk) (in rodents) and c-Cbl-interacting protein of 85kDa (CIN85) (in human), also known as SETA and CD2BP3 (alias Ruk/CIN85) was cloned and characterized independently by three research groups and later shown to modulate EGFR functions (5–7). Ruk/CIN85 belongs to an evolutionarily conserved family of SH3-containing proteins (8) characterized by the presence of three N-terminal SH3 domains (A, B and C) followed by a proline-rich region and a C-terminal coiled-coil domain. In addition to EGFR regulation, various isoforms of Ruk/CIN85 (9) were found to play important roles in a plethora of processes such as apoptosis (5,10,11), membrane trafficking (12–16), rearrangement of actin cytoskeleton and cell adhesion (17,18).

The possible involvement of Ruk/CIN85 in the process of carcinogenesis is not well defined. Ruk/CIN85 was originally supposed to act as tumor suppressor because it was found to inhibit PI3K in primary neurons (5) and to downregulate receptor tyrosine kinase signaling (12,19–21). As a modulator of EGFR function it would be expected that Ruk/CIN85 plays a role in breast carcinogenesis. However, no data showing changes in the expression of Ruk/CIN85 in breast adenocarcinoma samples have been reported so far. Therefore, it was the aim of this study to investigate the expression of Ruk/CIN85 in patients’ conditionally normal breast tissue and invasive breast carcinomas and to unravel a mechanism by which Ruk/CIN85 could affect tumorigenesis. In this study we demonstrate that Ruk/CIN85 is upregulated in breast adenocarcinoma and adenocarcinoma metastases. We further show that Ruk/CIN85 deficient and weakly invasive breast adenocarcinoma cells can be converted to a more malignant phenotype upon increased expression of Ruk/CIN85 and that this conversion is due to the activation of the Src/Akt but not ERK1/2 signaling pathway. These data suggest that Ruk/CIN85 may be a potential therapeutic target in breast cancer.

Materials and methods

Abbreviations: CIN85, c-Cbl-interacting protein of 85 kDa; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MTTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI3K, phosphatidylinositol-3’ kinase; Ruk, regulator for ubiquitin kinase; SETA, SH3 domain-containing expressed in tumorigenic astrocytes; SH3, Src-homology type 3 domain; SH3KBP1, SH3 domain kinase binding protein 1.

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cancer. These patients were asked to provide the tissue samples and to sign an informed-consent form.

Immunochemistry

Immunohistochemical evaluation of Ruk/CIN85 expression was performed using nine adjacent conditionally normal breast tissue samples, nine infiltrating ductal carcinomas and six infiltrating lobular carcinomas of the breast and three lymph node metastases. Tissue samples were fixed immediately after surgical removal in 10% buffered formalin and embedded in paraffin. Five micrometer thick sections were cut from paraffin blocks, mounted on poly-L-lysine-coated glass slides and dried overnight at 37°C. Slides were deparaffinized in xylene and then rehydrated through graded ethanol solutions. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide. Antigen retrieval was achieved by microwave heating in 10 mM citrate buffer (pH 6.0) for 15 min (3 x 5 min) at 600W. To prevent non-specific binding of primary antibodies, Biotin blocking system (DAKO) was used. The affinity purified polyclonal antibodies to the C-terminal region and affinity purified monoclonal antibody to the SH3A domain of Ruk/CIN85 protein (clone MISH-A1) were already described (22) and applied in 0.1% bovine serum albumin in Tris-buffered saline overnight at 4°C. Peroxidase labeling with the Vectastain ABC Kit (Vector Labs) and diaminobenzidine as chromogen was processed according to the manufacturer’s instructions. Sections were counterstained with hematoxylin and mounted with coverslips. Negative controls were generated by substituting primary antibodies with rabbit or mouse IgGs in 0.1% bovine serum albumin in corresponding dilutions. Microscopic semiquantitative analysis of staining was performed in high power fields under ×400 magnification (Axioplan, Carl Zeiss, Germany) and scored as follows: no staining—0; weak staining—1; moderate—2; strong staining—3.

Cell culture

Human breast adenocarcinoma MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 μM penicillin and 100 units/ml penicillin. For time-course studies of ERK1/2, Src and Akt activation, MCF-7 cells were plated on 6 cm dishes and cultured for 2 days in full serum. Before EGF treatment, the cells were cultured for 24 h in starvation medium (DMEM containing 0.1% FCS). The cells were treated for 1, 5, 10 or 30 min with either sterile phosphate-buffered saline (control) or EGF (100 ng/ml).

Generation of MCF-7 sublines that stably express wild-type Ruk/CIN85

MCF-7 cells, which have almost undetectable levels of Ruk/CIN85, were transfected either with pRcCMV2-Ruk (5) or empty vectors by calcium phosphate precipitation. After transfection cells were subjected to selection with 2 mg/ml geneticin (G418) (Sigma, St. Louis, MO). Geneticin-resistant cells were subcloned and screened for expression of Ruk/CIN85 by western blot analysis. Four independent sublines of MCF-7 cells that stably express untagged Ruk/CIN85 were selected. MCF-7 cells transfected with empty vector and cultured in the presence of geneticin (G418) were used as control.

Protein preparation and western blot analysis

MCF-7 cells were scraped in lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM o-vanadate, 50 mM NaF, 2 mM β-glycerophosphate, complete protease inhibitor cocktail tablet (Roche)], mechanically triturated through a 1 ml syringe, kept on ice for 10 min and centrifuged at 14 000 g for 20 min at 4°C. Frozen tissue samples were triturated in liquid nitrogen and extracted with lysis buffer. After centrifugation of lysates, protein content in supernatants was determined using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL).

Proteins (30 μg per sample) were separated by electrophoresis on 10–12.5% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with either monoclonal or polyclonal anti-Ruk/CIN85 (22), anti-ERK1/2 (Sigma), anti-phospho-ERK1/2 (Thr 202/Tyr 204) (Sigma), anti-Akt (Cell Signaling), anti-phospho-Akt (Ser473) (Cell Signaling), anti-phospho-Src (Tyr418) (Invitrogen), anti-EGFR (Cell Signaling), anti-β-actin (Sigma) or anti-β-actin (Sigma) antibodies overnight at 4°C. Appropriate secondary antibodies (peroxidase-conjugated IgG (Promega)) were used at 1:3000 dilutions. The enhanced chemiluminescence kit (Amersham Pharmacia Biotech) was used for detection. Densitometric analysis was performed using the Gel-Pro analyzer software (Media Cybernetics, Silver Spring).

Cell survival and proliferation assays

Cell growth and viability were assessed by trypsin blue dye exclusion and by a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. MCF-7 cells were plated on 6 cm dishes (4 x 10⁴ per dish) and cultured for 24–168 h, as indicated. The tryptisized cells were washed and stained with trypsin blue. To include unattached MCF-7 cells into the measurements, the culture media containing floating cells were centrifuged at 1000rpm for 3 min before trypsin blue staining. The total cell number and the number of live cells were counted in a Neubauer chamber. Cell survival was calculated as the ratio of live cells to the total number of cells. The MTT test was carried out in 24-well dishes (5 x 10⁴ cells per well) at 37°C for 2 h. Formation of MTT–formazane was determined spectrophotometrically at a wavelength of 570 nm using an absorbance microplate reader Quant (BioTEK).

Assay of anchorage-independent growth

Anchorage-independent cell growth was determined by soft agar colony formation assay. Equal numbers of cells at a density of 10⁷/ml in semisolid medium (33% agar in DMEM containing 10% FCS) were added to a 3.5 cm plate containing 0.5% agar in 10% FCS-containing DMEM. The number of colonies formed in agar was recorded 2 weeks after plating the cells.

Wound healing assay

MCF-7 cells were plated into 6-well dishes and grown to 80% confluence in DMEM containing 10% FCS. For the next 24 h monolayers were grown in starvation medium (0.1% FCS) without phenol red. Then cells were grown in the same media either in the presence of EGF (100 ng/ml) or vehicle for 3 days. Wounding was induced by manually scratching the cell monolayer with a pipette tip. Following wounding, the cell culture media was replaced with the same media containing 100 ng/ml EGF and 1 μM mitomycin C (Sigma) to eliminate effects of cellular proliferation. The wound closure was monitored 24 h later by phase-contrast microscopy. The distance migrated by the cells was measured using image-processing software (QuickPHOTO Camera 2.2).

Transwell migration assay

Cell motility assay was performed using 24-well cell culture inserts with 8 μm pores (Becton Dickinson) in which the lower surface of the membrane was coated with collagen type I (10 μg/ml) in order to facilitate cell migration (23). For these assays, the cells were treated with EGF for 3 days. Specific inhibitors of PI3K (LY294002, 10 μM), Src (PP2, 1 μM) or MEK (U0126, 10 μM) were added, when indicated, 20 min before EGF. Then, 3 x 10⁴ cells were seeded on the upper wells of the 24-well chambers in the presence of 0.1% serum. The lower wells were filled with medium containing 5% FCS and (when indicated) 100 ng/ml EGF. After incubation for 12 h, cells that migrated out onto the lower surface of membranes were fixed in 4% paraformaldehyde. These cells were stained with 1% crystal violet and counted in five random fields (more than 100 cells were scored in each experiment).

Statistics

Statistical analysis was carried out using SPSS 12.0 software. Paired and unpaired Student’s t-tests were performed and the difference was considered to be significant when the P-value was ≤0.05.

Results

Expression of Ruk/CIN85 is induced in breast adenocarcinoma

In order to find out whether Ruk/CIN85 expression is changed in breast adenocarcinoma cells, we performed immunohistochemical analyses with surgical samples from breast cancer patients using monoclonal anti-Ruk/CIN85 antibody that recognizes only full-length form of Ruk/CIN85 (Figure 1A, a–f). A positive staining of low intensity was found in ductal and lobular epithelium in almost all non-cancerous adjacent tissue samples (Figure 1A, b). The intensity of staining was significantly higher in cancer tissues including lymph node metastases in comparison with normal breast tissues (Figure 1A, c–e). The similar results were obtained using polyclonal antibodies to the C-terminus of Ruk/CIN85, recognizing additional splice variants with intact C-terminal regions (Supplementary Figure 1A, available at Carcinogenesis Online). The highest expression of Ruk/CIN85 was found in intravascular tumor embols (Figure 1A, f). Among stromal components, mast cells demonstrated comparatively high Ruk/CIN85 staining compared with other cell types.

An overall semiquantitative analysis showed that among 15 adenocarcinoma sections, 80% demonstrated either moderate (2) or strong (3) Ruk/CIN85 staining whereas only one sample had no staining at all. Simultaneously, all non-cancerous breast tissue sections received scores 0 or 1 for Ruk/CIN85 expression (Supplementary Table 1, available at Carcinogenesis Online). We further measured the expression levels of Ruk/CIN85 by western blotting using monoclonal anti-Ruk/CIN85 antibodies in both
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**Rukl/CIN85 is overexpressed in human breast adenocarcinoma and lymph node metastases.** (A) Expression of Ruk/CIN85 in non-cancerous breast epithelium (b), breast adenocarcinoma (c and d), lymph node metastases (e) and intravascular tumor embols (f), detected by immunohistochemistry using the monoclonal anti-Ruk/CIN85 antibody MISh-A1. A negative control stain is shown (a). (B) Western blot analysis of Ruk/CIN85 expression in adenocarcinoma samples in comparison with non-cancerous breast tissue from the same patients. β-Actin was used as a loading control. c—non-cancerous (control) tissue sample, t—adenocarcinoma tumor sample from the same patient. (C) Results of western blotting were quantified by densitometry. The level of Rukl/CIN85 expression detected with MISh-A1 in non-cancerous tissue was set to 1. Data represent mean ± SEM of 31 samples, *P ≤ 0.01.

**Ruk/CIN85 promotes cell motility**

In order to investigate whether Ruk/CIN85 expression influences motility of MCF-7 cells, transwell migration and wound closure assays were performed. Although migration of D4 cells through the transwells did not differ significantly from the control, the migration of D4 cells was increased by about 50% when compared with control cells (Figure 3A and 3B). In a wound closure assay the motility of both D4 and G4 cells was significantly higher than that of control cells (Figure 3C). These data suggest that the differences in cell growth dynamics could be attributed to Ruk/CIN85-dependent changes in the proliferative capacity and not to the differences in their viability.

**Ruk/CIN85 expression changes cell growth dynamics properties**

To analyze mechanismal aspects by which Ruk/CIN85 would change biological properties of breast adenocarcinoma cells we generated stable clones of Ruk/CIN85-expressing MCF-7 cells. These cells appear to be a valid model for such experiments because they are weakly invasive and deficient of Ruk/CIN85. Four different sublines of MCF-7 cells expressing Ruk/CIN85 were established and designated D4, G4, G9 and G10. The highest level of Ruk/CIN85 expression was detected in G4 cells whereas the lowest Ruk/CIN85 level was observed in D4 cells (Figure 2A). These two cell lines were mainly used in the further experiments. The data concerning G9 and G10 clones, that do not differ significantly from the results obtained with G4, are not shown.

First, we investigated whether Ruk/CIN85 expression was associated with variations in cell growth rates. We found that expression of Ruk/CIN85 altered the dynamics of cell growth as indicated by the number of cells. This was most visible 96 h after plating when control cells attained a stationary growth phase (Figure 2B). At the same time (96 h) the number of D4 cells was about 30% lower when compared with the control. This appeared to be a Ruk/CIN85 dose-dependent effect since the subline G4 with the highest levels of Ruk/CIN85 showed about 50% reduction in cell number (Figure 2B). These data are consistent with the results of the MTT assay that showed 45 ± 12% and 26 ± 4% reduction in metabolic activity of G4 and D4 cells, respectively, when compared with control MCF-7 cells 72 h after plating (n = 4, P ≤ 0.05).

In order to explain the observed differences, we first investigated cell viability using the trypan blue dye exclusion method. The viability of control cells was slightly higher as compared with Ruk/CIN85-expressing cells up to 96 h in culture and slightly lower from 96 to 168 h although these differences were not significant (Figure 2C). These data suggest that the differences in cell growth dynamics could be attributed to Ruk/CIN85-dependent changes in the proliferative capacity and not to the differences in their viability.

**Rukl/CIN85 alters cell adhesion properties**

During the course of the experiments we observed that a high number of Ruk/CIN85-expressing cells were present in suspension and many of these cells remained viable. The viability of suspension G4 cells (56 ± 4.7%) was slightly but not significantly higher as compared with control (43 ± 5.8%) and D4 (42 ± 7.4%) cells. The number of floating cells was about 2.5-fold higher for the D4 and about 3-fold higher for the G4 cells when compared with the control cells (Figure 2D). These findings indicate that cells expressing Ruk/CIN85 possess decreased adhesive properties.

The ability of the cells to proliferate unattached to a surface was then investigated by using a soft agar colony formation assay. It was found that cells expressing Ruk/CIN85 formed significantly more colonies 2 weeks after plating than the control cells; the G4 subline formed 380% and the D4 subline 125% colonies (Figure 2E and 2F). Thus, the ability of Ruk/CIN85-expressing cells to grow better in semisolid agar indicates that they acquire a more transformed cellular phenotype.
cells (by about 60 and 40%, respectively) (Figure 3C and 3D). Thus, these data indicate that Ruk l/CIN85 can interfere with mechanisms that control migration of breast cancer cells.

Ruk l/CIN85 affects ERK1/2, Akt and Src activity

Hyperactivation of the ERK1/2, Akt and Src kinases is often observed in breast cancers and is known to be among the factors responsible for the resistance to endocrine and HER2-targeted therapies (24). In addition, breast carcinomas and a number of other solid tumors have been associated with an impaired EGF-induced signaling (25). Therefore, we investigated phosphorylation levels of ERK1/2, Akt and Src in control and Ruk l/CIN85-expressing cells under basal conditions and after stimulation with EGF. We observed that although basal ERK1/2 phosphorylation was almost undetectable in control and in Ruk l/CIN85 cells, both Akt and Src phosphorylation was constitutively increased in the cells that express Ruk l/CIN85 (Figure 4A–C).

When cells were treated with EGF for 1, 5, 10 and 30 min we found that the Ruk l/CIN85 expressing subline G4 responded faster than the control cells to EGF treatment (Figure 4A–C). In the G4 cells, ERK1/2 became activated already within 1 min after EGF addition. The kinase remained active up to 30 min after EGF treatment whereas in control cells ERK1/2 activity was lost after 30 min. Further, the subline G4 displayed a more rapid and prolonged EGF-dependent activation of Src and Akt when compared with control cells. This appeared to be Ruk l/CIN85 dose-dependent since the dynamics of ERK1/2, Akt and Src activation in the D4 subline with low levels of Ruk l/CIN85 expression did not differ significantly from the control cells (Figure 4A–C). The changes in EGF-induced activation of intracellular signaling pathways observed in our study could not be attributed to the changes in EGFR expression since EGFR levels were found to be similar in control and Ruk l/CIN85-expressing cells (data not shown). Further, we could also show that Akt phosphorylation was due to Src activity since the Src inhibitor PP2 blocked Akt phosphorylation in G4 cells (Figure 4D). These data indicate that Src is localized “upstream” of Akt in the EGF-induced signaling pathways in MCF-7 cells. Together, these
results demonstrate that Ruk/CIN85 leads to activation of the Src/Akt pathway and influences EGF-induced ERK1/2, Akt and Src phosphorylation in MCF-7 cells.

Ruk/CIN85-expressing cells lose sensitivity to EGF-induced cell motility but not proliferation

The obtained data concerning EGF-induced signaling suggest that expression of Ruk/CIN85 would lead to changes in MCF-7 cell responsiveness to EGF. It has been previously demonstrated that EGF treatment could increase proliferation and motility of MCF-7 cells via mechanisms dependent on stimulation of Raf/ERK and PI3K/Akt pathways (26,27). We found that the treatment of both control and Ruk/CIN85-expressing cells with EGF increased their proliferation by about 50% (Figure 5A). In contrast, when performing transwell migration assays we found that EGF increased migration only in control cells by about 40% whereas migration of D4 and G4 cells was not increased (Figure 5B and 5C). Similar data were obtained by using a wound closure assay where EGF induced the motility of control cells by about 2-fold, but did not significantly stimulate motility of the Ruk/CIN85-expressing cells (Figure 5D and 5E). Thus, expression of Ruk/CIN85 decreases sensitivity of MCF-7 cells to EGF-mediated induction of cell motility.

We further used the specific inhibitors of the PI3K (LY294002, 10 µM), Src (PP2, 1 µM) and MEK (U0126, 10 µM) in transwell assays to investigate the possible involvement of these pathways in Ruk/CIN85-dependent cell motility changes. Whereas U0126 treatment decreased basal motility both in control and in Ruk/CIN85-expressing cells, PP2 and LY294002 decreased motility only in G4 cells (Figure 6). However, neither inhibitor restored sensitivity of Ruk/CIN85-expressing cells to EGF-induced motility. Together, these data show that basal Ruk/CIN85-induced changes in cell motility are due to Src/Akt activation and that this renders the cell motility insensitive to EGF.

Discussion

In this study we showed that tumor samples from patients with invasive breast adenocarcinomas contained high levels of the adapter protein Ruk/CIN85 indicating that it possibly contributes to malignancy and metastasis. Expression of Ruk/CIN85 in Ruk/CIN85-deficient MCF-7 breast adenocarcinoma cells lead to a conversion of these cells to a more transformed malignant phenotype. The involvement of Ruk/CIN85 in the process of tumorigenesis was so far not well defined. A Ruk/CIN85 isoform SETA (SH3 domain-containing gene expressed in tumorigenic astrocytes) was originally identified as a protein associated with malignancy in astrocytes (7). Being barely detectable in the normal adult rat or human brain, SETA mRNA was found in about 50% of the human gliomas tested, in a rat glioma generated by ethylnitrosourea treatment and in a number of glioma-derived cell lines (7,28). Though SETA was shown to be selectively expressed only in tumorigenic astrocytes derived from p53 knockout mice, the molecular mechanisms of its involvement in the process of glioma formation were not resolved. In the recent years we and two other groups reported increased expression of Ruk/CIN85 in several human malignancies such as melanoma (22), head and neck squamous cell carcinomas (29) and cervical carcinoma (30). Our present data on increased expression of Ruk/CIN85 in several human malignancies such as melanoma (22), head and neck squamous cell carcinomas (29) and cervical carcinoma (30). Our present data on higher Ruk/CIN85 expression in breast adenocarcinoma and positive contribution of Ruk/CIN85 to the development of breast cancer are therefore in line with these studies. The results of our study are also consistent with the observation that invasive breast cancer cells as compared with weakly or noninvasive breast cancer cell lines generally express higher amounts of Ruk/CIN85 (31). Moreover, Ruk/CIN85 was also shown to be involved in the maintenance of breast cancer cell invasiveness in several highly invasive breast adenocarcinoma cell lines (31). It was shown that siRNA-mediated silencing of Ruk/CIN85 inhibited MDA-MB-231, MDA-MB-435s and to a lesser extent Hs578T breast cancer cell chemoinvasion in a Matrigel assay (31). It has been argued that interaction of Ruk/CIN85 with a
Fig. 4. Ruk/CIN85 enhances ERK1/2, Akt and Src activation. Control MCF-7 and Ruk/CIN85-expressing D4 and G4 subline cells were treated with EGF (100 ng/ml) for the indicated periods of time. The phosphorylation of Akt, ERK and Src was measured by western blot analysis with phosphospecific antibodies. (A) The ratio of phosphorylated ERK1/2 (pERK) to total ERK1/2 in untreated control cells was set equal to 1. Representative blots are shown in the right panels. Data are expressed as mean ± SEM of four independent experiments, *P ≤ 0.05 as compared with control. (B) The ratio of phosphorylated Akt (pAkt) to total Akt in untreated control cells was set equal to 1. Representative blots are shown in the right panels. Data are expressed as mean ± SEM of five independent experiments, *P ≤ 0.05 as compared with control. (C) The ratio of phosphorylated Src (pSrc) to β-tubulin as loading control in untreated control cells was set equal to 1. Representative blots are shown in the right panels. Data are expressed as mean ± SEM of three independent experiments, *P ≤ 0.05 as compared with control. (D) The cells were treated with EGF as before either in the presence or absence of Src inhibitor PP2 (1 µM). Src and Akt phosphorylation was detected by western blot analysis.
multidomain Arf GTPase activating protein AMAP1 was important for the formation of invadopodia that allow aggressive cancer cells to degrade extracellular matrix and to spread through surrounding tissues (31). Moreover, several proteins recently identified as novel putative interaction candidates for the SH3 domains of Ruk/CIN85, including N-WASP, the N-WASP-interacting adaptor molecule WIP, Tks4 and dynamin, have a well-documented role in the biogenesis of invadopodia (16,32). It has been suggested that Ruk/CIN85 might control invadopodia biogenesis directly via the augmentation of actin assembly (16). It might be also suggested that ASAP1 sequences Ruk/CIN85 from interacting with ubiquitin-binding/lysosomal sorting proteins, or from interacting with the EGFR, both of which may lead to enhanced recycling of EGFR, that potentially contributes to the invasiveness of breast cancer cells. Interestingly, we recently demonstrated that Ruk/CIN85 expression led to stabilization of HIF-1α (33) that is known to promote tumorigenesis (34).

Beside the studies demonstrating a stimulatory contribution of Ruk/CIN85 in the carcinogenesis there are also data indicating the role of Ruk/CIN85 as a putative tumor suppressor. Isoforms of this protein have been shown to increase susceptibility of astrocytes to UV irradiation-induced apoptosis and of T-leukemia cells to TNF-α-induced apoptosis (10,35), to attenuate PI3K activity by interaction with its regulatory p85α subunit stimulating apoptosis in primary neurons (5), to downregulate receptor tyrosine kinase signaling by clathrin-mediated internalization of activated EGF, hepatocyte growth factor and platelet-derived growth factor receptors (12,19,20,36). On the other hand, the data concerning the role of Ruk/CIN85 in the internalization of growth factor receptors through clathrin-coated pits are somewhat contradictory (15,37,38). Thus, additional studies are necessary for better understanding of the role of Ruk/CIN85 and other Ruk/CIN85 isoforms in the regulation of different aspects of carcinogenesis in various cell culture models.

The Raf/ERK and PI3K/Akt signal transduction pathways as well as Src family kinases are known to be responsible for the regulation of multiple physiological processes in breast cancer cells (2,39,40). We found that the basal levels of Src and Akt activation in Rukl/CIN85-expressing cells were higher than in the control (Figure 4). These data together with the observation that inhibitors of Src and PI3K (PP2 and LY294002) decreased basal cell motility of Rukl/CIN85 expressing but not control cells suggest that the changes of MCF-7 transforming potential observed in our study were due to the constitutive activation of these kinases. The exact mechanism of Rukl/CIN85 involvement in these processes remains to be elucidated though Src was shown to be one of Rukl/CIN85 binding partners (5,41).

In addition to the changes in basal activity of the signal transduction machinery, the impaired activation of signaling networks upon growth factors treatment is characteristic for the process of carcinogenesis. In particular, there is a well-established correlation Fig. 5. Rukl/CIN85-expressing cells display no sensitivity to EGF-induced motility. (A) Control MCF-7 and Ruk/CIN85-expressing D4 and G4 subline cells were plated in 24-well dishes and treated either with EGF (100 ng/ml) or vehicle for 72 h. Then, metabolic activity was assayed by a MTT test. Values are percent of corresponding untreated controls. Data are expressed as mean ± SEM of four independent experiments. (B) Control MCF-7 and Ruk/CIN85-expressing D4 and G4 subline cells were treated either with EGF (100 ng/ml) or vehicle for 3 days and analyzed in a transwell migration assay. The number of migrated untreated cells was set equal to 100% for each subline. Data are expressed as mean ± SEM of four independent experiments. *P ≤ 0.05 Rukl/CIN85-expressing cells compared with control cells. (C) Representative photographs of the filters are shown; original magnification is 400. (D) Control MCF-7 and Ruk/CIN85-expressing D4 and G4 subline cells were treated either with EGF (100 ng/ml) or vehicle for 72 h and then a wound healing assay was performed. The average distance moved by untreated cells was set equal to 100% for each subline. Data are expressed as mean ± SEM of four independent experiments. *P ≤ 0.05 Rukl/CIN85-expressing cells compared with control cells. (E) Representative photographs of wound areas either immediately or 24 h after scratching are shown; original magnification is 200, untr.—EGF-untreated cells.
between upregulation of EGF-induced intracellular signaling pathways and a poor prognosis in breast cancer patients (42,43). It was also shown that in head and neck squamous cell carcinoma lines Ruk/CIN85 increases proliferation through potentiation of EGFR-mediated Ras/ERK pathway activation (29). Therefore, we investigated the effect of stable Ruk/CIN85 expression on ERK1/2, Src and Akt activation upon EGF treatment and found that their activation following addition of EGF was faster and more prolonged than in the control cells. It was previously demonstrated that treatment of MCF-7 cells with EGF induced a modest effect on cell proliferation and a pronounced effect on cell motility (26, 44–46). Our present results are consistent with these observations. In our study, EGF-induced stimulation of MCF-7 cell motility but not proliferation was abolished in Ruk/CIN85-expressing cells. Since high and sustained levels of Akt and ERK1/2 activation are associated with high metastatic potential of breast cancer cells (47,48), it was somehow unexpected that the increased duration of Raf/ERK and PI3K/Akt pathways activation following EGF treatment of Ruk/CIN85-expressing cells was accompanied by the decrease in EGF-stimulated motility. A recent study demonstrated that in contrast with Ruk/CIN85, expression in MCF-7 cells of the adaptor/scaffold protein caveolin-1 led to an increase in basal and EGF-induced Akt (but not ERK) phosphorylation associated with enhanced EGF-dependent proliferative and motility rates (46). It should be noted, however, that in our study inhibition of Akt, Src and ERK phosphorylation did not restore sensitivity of Ruk/CIN85-expressing MCF-7 cells to EGF-induced motility. Therefore, it could be speculated that other components of intracellular signaling pathways are important for the effects of Ruk/CIN85 expression in EGF-treated cells observed in our study.

Taken together, we have demonstrated that Ruk/CIN85 levels are high in adenocarcinomas and lymph node metastases of breast cancer patients. To further define the role of Ruk/CIN85 in breast cancer progression we reconstituted high Rukl/CIN85 expression in breast cancer patients. To further define the role of Ruk/CIN85 in breast cancer progression we reconstituted high Rukl/CIN85 expression in weakly invasive MCF-7 breast adenocarcinoma cells. The expression of Ruk/CIN85 resulted in decreased cell adhesion and enhanced anchorage-independent growth and motility. These changes are consistent with the features of cells with a more aggressive phenotype. Moreover, Ruk/CIN85-expressing cells display changes in Akt, Src and ERK1/2 activation upon EGF treatment, and show lower EGF-induced motility. Altogether, these findings define a new role for Ruk/CIN85 in the progression of breast cancer.

Supplementary material

Supplementary Figure 1 and Table 1 can be found at http://carcin.oxfordjournals.org/

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References


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