Oncogenic synergism between ErbB1, nucleolin and mutant Ras

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ABBREVIATIONS

Abs, antibodies; DMEM, Dulbecco’s modified Eagle medium; EGF, Epidermal growth factor; FTS, S-trans, trans-farnesylthiosalicylic acid; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; NCL, nucleolin; PBS, phosphate buffered saline; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
ABSTRACT

Alterations in the ErbB family of growth factor receptors, their signaling components and mutational activation of Ras proteins are major contributors to malignant transformation. Recently, mutant Ras was shown to be capable of activating ErbB receptors in a ligand-independent manner. Further, it was observed that nucleolin, a transcriptional regulator and ribosome biogenesis factor, can bind both K-Ras and the cytoplasmic tail of ErbB receptors to enhance ErbB receptor activation. However, the functional significance of these interactions to cancer pathogenesis has not been probed. Here we show that endogenous nucleolin interacts simultaneously in vivo with endogenous Ras and ErbB1 (EGFR) in cancer cells. The C-terminal 212 amino acids of nucleolin were determined to be sufficient to interact with ErbB1 and with all Ras protein isoform (H-, N- and K-Ras). Nucleolin partially colocalizes with Ras at the plasma membrane. Moreover, activated but not wild type Ras facilitates nucleolin interaction with ErbB1 and stabilizes ErbB1 receptor levels. Most importantly, these three oncogenes synergistically facilitate anchorage-independent cell growth in vitro and tumor growth in vivo. Our findings suggest strategies to target nucleolin as a general approach to inhibiting ErbB- and Ras-driven cancers.
Introduction

Receptor tyrosine kinases (RTK) regulate cell proliferation and differentiation when activated upon binding to their respective growth factor ligands, and when deregulated may lead to the development of cancer (1, 2). The ErbB subfamily of RTKs contains four members: ErbB1 (EGFR, HER1), ErbB2 (Neu, Her2), ErbB3 (HER3), and ErbB4 (HER4), which have been strongly implicated in the development of human cancers (2). Activation of ErbB receptors induces receptor dimerization, which transmits a signal across the plasma membrane and activates the intracellular tyrosine kinase domain (3, 4). This in turn leads to trans-autophosphorylation on tyrosine residues that function as docking sites for signal transducing molecules (5). Among the most prominent signaling pathways activated by ErbB receptors is the Ras/MAPK cascade (6). Activated Ras can induce the release of growth factors including EGF-like ligands such as amphiregulin, TGFα, HB-EGF and NRG (7, 8), thereby inducing receptor phosphorylation via an autocrine or paracrine loop. Constitutively activated Ras can also induce ErbB phosphorylation in a ligand-independent manner (9). Ras signaling is aberrantly activated in a large fraction of human tumors (1, 10). Because Ras signaling represents a convergence point for numerous diverse extracellular signals, Ras and its effectors are appropriate targets for therapeutic intervention. Abnormal function of the ErbB receptors and their ligands is also involved in human cancer and these receptors are currently targets of an increasing number of cancer drugs (2, 11).
Recently we demonstrated that ErbB1 interacts with the multifunctional protein, nucleolin (12), a ubiquitously expressed acidic phosphoprotein with key functions in transcription and in the synthesis and maturation of ribosomes (13, 14). Nucleolin is thus involved in critical aspects of transcriptional regulation, cell proliferation, and cell growth (15, 16). Nucleolin was originally identified as a nuclear protein localizing primarily to the nucleoli, but is now appreciated to undergo nuclear-cytoplasmic shuttling and to also be present on the cell surface of some types of cells (14, 17, 18). Inhibition of cell-surface nucleolin and nucleolin activities suppresses cell and tumor growth (13, 19-21) in breast, prostate and glioma cell lines, which also express high levels of ErbB receptors and/or activated Ras protein. An aptamer targeting nucleolin, AS1411, is in phase II clinical trial for relapsed/refractory acute myeloid leukemia (22).

We recently identified non-nuclear nucleolin as an ErbB receptor-interacting protein (12). This interaction leads to receptor dimerization and activation as well as to colony growth in soft agar. The ErbB1 juxtamembrane region containing the nuclear localization sequence (NLS), which is important for ErbB1 kinase activation (23), is also important for nucleolin-ErbB1 interaction and for nucleolin-induced ErbB1 dimerization and activation (24). In addition we demonstrated that this effect is mediated by the 212 C-terminal amino acids of nucleolin (24). Thus it was suggested that the crosstalk between nucleolin and ErbB proteins might be related to tumor growth.
In the present study we demonstrate a crosstalk between nucleolin, ErbB1 and Ras proteins. All three typical Ras isoforms (H-, K- and N-Ras) interact with nucleolin. The 212 C-terminal amino acids of nucleolin are sufficient for this interaction and colocalize with Ras at the plasma membrane. Moreover, we demonstrate that GTP-bound Ras strongly enhances ErbB1/nucleolin interaction. Finally, we demonstrate that expression of activated H-Ras(G12V), nucleolin and ErbB1 synergistically enhance cell transformation, as evidenced by increased colony formation in soft agar and increased tumor volume in nude mice.
Materials and Methods

Materials and Buffers

Human recombinant EGF was purchased from Boehringer Mannheim. Polyclonal anti-ErbB1 antibodies (Abs), monoclonal anti-phosphotyrosine, anti-GFP and antinucleolin were purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal anti-pan-Ras and polyclonal anti-Ras were purchased from Calbiochem. Monoclonal anti p-Erk were purchased from New England Biolabs inc. Anti-β actin and anti-H-Ras monoclonal antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal anti β-tubulin and all other reagents were from Sigma. GRO (AS1411) aptamer and CRO (inactive oligomer) were synthesized as previously described (13, 22). Salirasib (FTS), a farnesylcysteine mimetic, was a gift from Concordia Pharmaceuticals (Fort Lauderdale, FL) and was prepared for experiments as described previously (25). HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1.5 mM MgCl₂, 10% glycerol, 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin.

Cell Lines

HEK-293, HEK-293T, MDCK and U87 cell lines were grown in Dulbecco’s modified Eagle’s (DMEM) and DLD1 cell line was grown in RPMI-1640, supplemented with 10% fetal bovine serum (FBS). Cells were transfected using jetPEI (Polyplus...
Transfection, USA) or using calcium phosphate (CaPO4) precipitation. Cell lysates were prepared 48 h following transfection as described. Preparation of HEK-293 cells stably expressing the ErbB-1 receptor was previously described (24). Expression vectors GFP-ErbB1, GFP-H-Ras (12V) or pcDNA3-H-Ras(12V) and DsRed-nucleolin were introduced by JetPeI transfection into MDCK cells either alone or together. The neomycin (G418)-resistant colonies were examined for ErbB-1, Ras or nucleolin expression and several colonies (3 clones from each combination) were selected for further analysis. NIH3T3 were obtained from ATCC and routinely grown in a humidified incubator at 37°C with 5% CO2. Cells were grown in DMEM supplemented with 10% bovine serum (BS; Life Technologies No. 16170078), 62.5 μg/mL penicillin and 100 μg/mL streptomycin (Hyclone Laboratories, Logan, Utah). NIH3T3 were transfected with the vector pZIP, or pZIP-H-Ras (12V), using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s directions, and geneticin-resistant cells were selected to be examined by western blot.

DNA Constructs

GFP-ErbB1 was provided by Marcello Erlich, Tel Aviv University, Israel. Expression vectors for pEGFP-nucleolin, DsRed-nucleolin (DsRed-NCL) and DsRed-nucleolin mutants were previously described (12, 24). Flag-nucleolin expression vectors were generously donated by both James A Borowiec (New York University, USA) and Paula J Bates (University of Louisville, USA). N-Ras and N-Ras(13V) expression
vectors were previously described (26). GFP-K-Ras, GFP-K-Ras(12V), GFP-H-Ras and GFP-H-Ras(12V) were previously described (27).

**Lysate Preparation, Immunoprecipitation and Immunoblotting**

Cells were exposed to the indicated stimuli and solubilized in solubilization buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added to cell lysates. For other experiments, Abs were first coupled to anti-mouse IgG agarose for 1 h at RT; alternatively, anti-Flag M2 agarose beads (Sigma) were used. Proteins in the lysate supernatant were precipitated with aliquots of the beads-antibody complexes for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, resolved by SDS-polyacrylamide gel electrophoresis (28) and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 1 h in TBST buffer (0.02 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 6% milk, blotted with 1 µg/ml primary Abs for 2 h, followed by 0.5 µg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp, UK).

**Imaging**

HEK-293T cells were plated on glass coverslips placed in 6 well plates at a density of 2 x 10^5 cells per well. Cells were transfected with or without pCDNA3-ErbB1 with GFP-KRas (12V) or GFP-HRas (12V) and either DsRed, DsRed-NCL, DsRed-N-ter or DsRed-212 nucleolin expression vectors. After 48 h, cells were fixed in 4%
paraformaldehyde at room temperature for 30 min. For triple staining, fixed slides were immunostained with rabbit anti-ErbB1 antibodies (1:100) followed by Alexa fluor 405-labeled donkey anti rabbit secondary antibodies (1:1000; Invitrogen, Eugene, OR) as previously described (29). Cells were examined by fluorescent microscopy at 63x magnification with Zeiss 510 META confocal microscopy.

**Soft Agar Assay**

Cells were seeded at a density of 1,000 cells/well in 96 well plates in DMEM containing 10% FBS. The cells were mixed with 0.05 ml (per well) of 0.33% Noble agar, and the mixture was poured onto a layer of 0.05 ml 1% Noble agar in DMEM containing 10% FBS. The upper layer of the agar was covered with 0.1 ml of culture medium. Assays were performed at least six times. The number of colonies with size above 0.01 mm² was estimated on day 8, using a binocular and light microscopy with the image analyzer program Image Pro-Plus. Results shown are the mean ± SD of 6 determinations from each of the clones used.

**Tumor Growth in Nude Mice**

MDCK cells harboring nucleolin, ErbB1, H-Ras(12V) and their combinations were implanted into nude mice. Nude CD1-Nu mice (25-30 g) were housed in barrier facilities on a 12-h light/dark cycle. Food and water were supplied ad libitum. On day zero, $2 \times 10^6$ cells of each clone in 0.1 ml of PBS were implanted subcutaneously just above the right femoral joint. Tumor volumes were then monitored on the indicated days as described previously (30).
RESULTS

Analysis of nucleolin/Ras interactions

In our previous reports we have demonstrated that nucleolin interacts with each of the four ErbB receptors and that this interaction has functional consequences, as it induces ligand-independent receptor activation (12). In related studies we found that constitutively active Ras also induces receptor phosphorylation in a ligand-independent manner (9). It was not known whether the two phenomena are related to each other, a possibility that might have a strong impact on cell transformation. We therefore investigated whether Ras can affect nucleolin-ErbB interaction. We first examined the ability of nucleolin to co-precipitate with Ras isoforms. Cells co-expressing Flag-nucleolin and GFP-tagged wild type (wt) or constitutively active H-Ras or K-Ras were lysed and subjected to immunoprecipitation of nucleolin using anti-Flag Abs, followed by immunoblotting for Ras with anti-GFP Abs (Figure 1A). As a control, cells were transfected with GFP-H-Ras(12V) and lysates were precipitated with anti-Flag Abs; no Ras precipitation was observed (Figure 1A). Similarly, cells expressing wt or constitutively active N-Ras were immunoprecipitated with anti-nucleolin Abs to pull down the endogenous nucleolin. The blots were then exposed to anti-Ras Abs (Figure 1B). As a control, lysates of cells expressing N-Ras(13V) were precipitated with beads in the absence of anti-nucleolin Abs (Figure 1B). As shown in Figures 1A-B all three Ras isoforms both in their wt and activated forms were
effectively co-precipitated with nucleolin, indicating that nucleolin can bind all Ras isoforms and that these interactions are independent of the GDP/GTP bound states of Ras.

We next postulated that if nucleolin mediates the effects of Ras on ErbB receptors then nucleolin will precipitate not only Ras but also ErbB. To this end we examined HEK-293 cells stably expressing ErbB1 and transiently expressing each of the wt or activated Ras isoforms. Cell lysates were subjected to immunoprecipitation with anti-nucleolin Abs and immunoblotted with anti-GFP, anti Ras or anti ErbB1 Abs as indicated. As a control cells were transfected with GFP only. We found that nucleolin precipitated each of the three Ras isoforms and also ErbB1 (Figure 2A-B). Importantly, nucleolin precipitated the ErbB1 receptor more effectively when co-expressed with each of the constitutively active Ras isoforms as compared with the wt proteins (1.38 ± 0.03, 1.57 ± 0.24 and 1.88 ± 0.23 fold for K-Ras, H-Ras and N-Ras, respectively, Figure 2A-B). These results indicate that GTP-bound Ras increases the interaction between nucleolin and ErbB1.

To further study the interaction between the three proteins, cell lysates from HEK-293 cells stably expressing ErbB1 and transiently expressing the K-Ras(12V) isoform were subjected to immunoprecipitation with anti-Ras Abs and immunoblotted with anti-ErbB1, anti-Ras or anti-nucleolin Abs (Figure 2C). As shown, Ras precipitated nucleolin as well as ErbB1. Taken together these results indicate that Ras interacts with nucleolin, as well as with ErbB1 and nucleolin interacts with Ras as well as with
ErbB1. Therefore it seems that there is a crosstalk between ErbB1, nucleolin and Ras, either directly or indirectly. We do not know whether these interactions occur simultaneously.

We then examined the interaction between ErbB1, nucleolin and Ras in human cancer cell lines without ectopic expression of any of these proteins. We used two cell lines: the colon cancer cell line DLD-1 that endogenously expresses ErbB1, nucleolin and mutant K-Ras(12V) and the glioblastoma cell line U87 that overexpresses ErbB1 and exhibits high Ras-GTP levels. Cell lysates were subjected to immunoprecipitation using anti-nucleolin Abs and immunoblotted with either anti-ErbB1 or anti-Ras Abs. As a negative control, lysates were precipitated with anti-GFP Abs. In both cell lines, nucleolin precipitated ErbB1 and Ras proteins (Figure 2D). These results support the conclusion that nucleolin interactions with ErbB1 and Ras occur in cancer cells expressing these proteins endogenously.

The 212 amino acid C-terminal region of nucleolin (212C-ter) interacts with Ras at the plasma membrane and is required for nucleolin/Ras interactions

We have shown previously that the C-terminal 212 amino acid region of nucleolin (212C-ter, scheme in Figure 3A), which contains the RNA binding domain 4 (RBD4, residues 498-645) and the glycine-arginine rich C-terminal region (GAR, residues 645-710), is both required and sufficient for nucleolin interaction with ErbB1 (24). To further examine the interaction between Ras and nucleolin we performed coimmunoprecipitation experiments on HEK-293T cells cotransfected with activated

13
Ras isoforms and GFP-nucleolin deletion mutants (Figure 3B-D). K-Ras(12V), H-Ras(12V) and N-Ras(13V) each immunoprecipitated effectively GFP-nucleolin 212-Cter and less effectively the individual GFP-tagged-GAR or -RBD4 domains. In contrast, the GFP-tagged-N-terminal mutant of nucleolin lacking RBD4/GAR (residues 1-498) did not precipitate with any of the Ras isoforms. These results suggest that the 212 C-terminal amino acid residues of nucleolin are both essential and sufficient for nucleolin-Ras interaction, as they are for nucleolin-ErbB1 interaction.

Next we examined whether nucleolin or its isolated 212 C-terminal amino acids colocalize with Ras at the plasma membrane, a major site of the interaction between nucleolin and ErbB1 (12) and of Ras (27). To this end we cotransfected HEK 293T cells with GFP-K-Ras(12V) or GFP-H-Ras(12V) and either pDsRed empty vector, pDsRed-nucleolin, pDsRed-212C-ter or pDsRed-N-ter (negative control). The subcellular localization of the labeled proteins was studied by dual fluorescent confocal microscopy. Consistent with our earlier studies (27), GFP-K-Ras(12V) was localized mainly to the plasma membrane but also to cytoplasmic vesicular compartments (Figure 4). Full-length pDsRed-nucleolin (RFP-NCL) was mainly localized to the nucleus, and also to the cytosol and plasma membrane, as expected (Figure 4). We observed partially overlapping localization of GFP-K-Ras and pDsRed-nucleolin at the plasma membrane. As predicted from its ability to co-immunoprecipitate Ras (Figure 3), a portion of DsRed-212C-ter overlapped with
GFP-K-Ras at the plasma membrane (Figure 4). Consistent with the fact that the nuclear localization sequences of nucleolin reside in its N-terminus, the majority of DsRed-212C-ter was not preferentially localized to the nucleus but was distributed throughout the nucleus and the cytoplasm, whereas DsRed-N-ter was localized exclusively to the nucleus, in which no GFP-K-Ras(12V) was detected. Thus, GFP-K-Ras(12V) and DsRed-N-ter showed no co-localization (Figure 4), as predicted by their lack of interaction when the cells were lysed (Figure 3). We saw similar overlapping localization of nucleolin with H-Ras (Figure 4). Next we examined the co-localization of the three proteins by performing a triple staining experiment. To this end we cotransfected HEK 293T cells with GFP-K-Ras(12V), pCDNA3-ErbB1 and either pDsRed empty vector, pDsRed-nucleolin, pDsRed-212C-ter or pDsRed-N-ter. The subcellular localization of the labeled proteins was studied by triple fluorescent confocal microscopy. As shown in, the ErbB1 (blue) colocalizes with GFP-K-Ras(12V) and DsRed-nucleolin or DsRed-212C-ter at the plasma membrane but not with pDsRed-N-ter (Figure 4 lower panel). Thus we conclude the three proteins colocalize at the plasma membrane, strengthening the notion of a crosstalk between them. These results also indicate that nucleolin 212 C-terminal amino acids are important for the interaction with both ErbB1 and Ras.

All Ras isoforms reduce EGF-induced reduction in the levels of ErbB1

Previously, we demonstrated that overexpression of nucleolin reduces EGF-mediated ErbB1 degradation (12). It is not known whether active Ras similarly causes such a
reduction. The strong association between nucleolin/ErbB1, and nucleolin/Ras prompted us to examine possible relationships between Ras and the levels of ErbB1. We thus examined next the effect of EGF on receptor levels in ErbB1-expressing HEK293 cells that were transfected with either GFP (control) or one of the activated Ras proteins: GFP-H-Ras(12V), GFP-K-Ras(12V) or N-Ras(13V). The transfectants were stimulated with EGF (100 ng/ml) for the indicated periods of time and cell extracts were subjected to immunoblot analysis with anti-ErbB1 Abs. In agreement with previous reports, EGF induced in the control cells (GFP transfectants) a time-dependent decrease in ErbB1 levels (Figure 5A). Each of the three activated Ras isoforms inhibited the reduction in ErbB1 receptor levels seen after EGF stimulation (Figure 5A). These results indicate that activated Ras isoforms affect receptor levels, either by reducing receptor degradation or by increasing receptor synthesis/transport.

Next we examined the effect of Ras or nucleolin inhibition on ErbB1 receptor levels. DLD1 cells were treated with FTS (Salirasib) or GRO (AS1411) or both for 48 h and then treated with EGF for the indicated time periods (Figure 5B). As shown inhibition of Ras with FTS and nucleolin with GRO resulted each in enhanced receptor degradation (Figure 5B). EGF-induced receptor degradation was enhanced even more in cells treated with both drugs. Similarly, these combined effects of the drugs were reflected in the downstream signaling to Erk (Figure 5B). Namely, inhibition of Ras and nucleolin together inhibited EGF-induced Erk activation even
more than each of the treatments alone. The crosstalk between Ras and nucleolin was also observed in cells expressing H-Ras(12V). Unlike growth of control cells, growth of H-Ras (12V) expressing cells was strongly inhibited by GRO (AS1411) but not by the control oligonucleotide (CRO) (Figure 5C).

**Co-expression of ErbB1, Ras and nucleolin facilitates cell transformation and tumorigenicity.**

We next examined whether the impact of nucleolin and Ras on ErbB1 is manifested in mediating cell transformation. To enable transformation and in-vivo experiments using stable cell lines, we prepared MDCK cells stably expressing either empty vector, nucleolin alone, nucleolin and ErbB1, H-Ras(12V) alone, nucleolin and H-Ras(12V), ErbB1 and H-Ras(12V) or the combination of all three proteins, and selected three individual clones from each transfection for further use. Levels of expression of each of the proteins in typical clones are shown in Figure 6A. We subsequently examined the possibility that the three proteins synergistically enhance cell transformation, by soft agar assays using the MDCK clones described above. Cells were plated in soft agar and maintained in culture for 8 days before quantitating the number and size of colonies able to grow in an anchorage-independent manner. Results of a typical experiment (Figure 6A) showed that, under the conditions tested, neither nucleolin nor H-Ras(12V) alone, nor nucleolin and ErbB1 together, induced the formation of colonies significantly higher than the background level of the small colonies seen with parental MDCK cells. In contrast,
expression of nucleolin and H-Ras(12V) or ErbB1 and H-Ras(12V) together induced the formation of much larger colonies than those formed by cells expressing each of the proteins alone (Figure 6, p< 0.001 and p< 0.01 respectively). Most importantly, MDCK cells overexpressing all three proteins formed a dramatically higher number of large colonies in soft agar as compared to clones expressing both nucleolin and H-Ras(12V) or ErbB1 and H-Ras(12V) (Figure 6, p<0.001).

We then examined whether these effects are mediated by nucleolin-212 Cter region which is important for the interaction with ErbB1 and Ras. We prepared clones that express stably either empty vector, nucleolin-212 Cter alone, nucleolin-212 Cter and ErbB1, H-Ras(12V) alone, nucleolin-212 Cter and H-Ras(12V), ErbB1 and H-Ras(12V) or the combination of all three proteins, and three individual clones from each transfection were selected (typical clones are shown in Figure 6B). As shown the 212-Cter was sufficient to mediate the anchorage independent growth effect when co-expressed with active Ras and ErbB1 (Figure 6B). These results strongly suggest that the combined expression of the three proteins synergistically enhance cell transformation and that the 212 C-terminal amino acids of nucleolin is sufficient.

To gain additional support for the possibility that nucleolin, H-Ras(12V) and ErbB1 synergistically enhance transformation, we performed experiments in nude mice. The mice were xenografted with each of the MDCK cell lines expressing the various combinations of the proteins. The following groups of mice (6 per group) received 2 x 10^6 cells subcutaneously in the flank: Mock; nucleolin alone; H-Ras(12V) alone;
nucleolin + ErbB1; nucleolin + H-Ras-(12V); or nucleolin, ErbB1 and H-Ras-(12V). Tumor volumes were monitored for 25 days following injection (Figure 7). Mock, nucleolin, or nucleolin + ErbB1 transfectants formed tumors that were only barely detectable and H-Ras(12V) or nucleolin + H-Ras(12V) transfectants formed relatively small tumors. In contrast, cells expressing nucleolin, ErbB1 and H-Ras(12V) formed large tumors (Figure 7, p<0.01) supporting the conclusion that they synergize in induction of cell transformation and that all three proteins are required for maximal transforming activity.

**DISCUSSION**

Nucleolin is a major component of the nucleolus and is also found in other cellular compartments (31). This key protein is involved in various aspects of ribosome biogenesis from transcriptional regulation to the assembly of pre-ribosomal particles. One study described interactions between nucleolin and K-Ras in the nucleolus (32). Other reports suggest that it could also play an important role in non-nucleolar functions (14). Recently we described a novel interaction between nucleolin and ErbB receptors at the plasma membrane and that this interaction enhances ErbB-mediated responses (12). In other experiments we showed that constitutively active Ras isoforms induce ErbB4 receptor phosphorylation in a ligand-independent manner, suggesting a direct intracellular positive control of Ras or its signals over ErbB4 (9). Together these studies raised the possibility of functional interactions between
nucleolin, ErbB receptors and Ras proteins. Here we demonstrate for the first time that crosstalk between the three proteins contributes to cell transformation. Our experiments show that nucleolin is capable of interacting with all three Ras isoforms, H-Ras, K-Ras and N-Ras. Previously, we found that H-Ras(12V) protein interacts specifically with galectin-1 (25, 33) and that K-Ras(12V) protein interacts specifically with galectin-3 (34). We also showed that these interactions are the driving forces that promote nanoclustering of active H-Ras (35) and of active K-Ras (36) proteins in the inner leaflet of the plasma membrane, processes known to promote robust digital signaling (37, 38). However, no escort proteins of N-Ras were reported. In the present study we have identified for the first time nucleolin as such an escort protein of N-Ras. Nucleolin is however less selective than galectins, first because it binds all Ras isoforms and second because it binds both the GDP-bound and GTP-bound forms of Ras. Thus, nucleolin/Ras interactions are independent of the GDP/GTP conformations of Ras proteins. Clearly then the interactions of Ras isoforms with nucleolin differ from those of H- and K-Ras proteins with galectins because only the latter depend on Ras-GTP (33, 34). However, the GTP-binding state of Ras is important for the interactions of ErbB1 and nucleolin, as discussed below.

The three highly homologous Ras proteins differ mainly in their hypervariable (hvr) region upstream of the common C-terminal farnesyl cysteine carboxymethylester (39-42). Because all three interact with nucleolin it is reasonable to assume that either the C-terminal farnesylcysteine and/or the domains upstream of the hvr that they share in
common may be involved in nucleolin/Ras interactions. Nucleolin was reported previously to interact with K-Ras4B but not with H-Ras in the nucleolus (32). In a study published during the final preparation of our manuscript it was reported that nucleolin from post-nuclear supernatants of BHK cells interacts with K-Ras but not H-Ras (43). The basis for this discrepancy is currently unclear. The latter study showed that a GFP-fusion protein containing only the hvr domain of K-Ras4B was sufficient for interaction with nucleolin, consistent with their and our findings that Ras/nucleolin interactions are not affected by the GTP-binding status of Ras. Additional experiments are required to determine which regions in Ras are critical for the interactions with nucleolin.

Using mutational analysis we found that the interaction between nucleolin and Ras is mediated specifically by the 212 C-terminal amino acids of nucleolin and not by the N-terminal domain of the protein. This interaction appears to occur predominantly at the plasma membrane since the 212 C-terminal nucleolin mutant overlaps there with K-Ras(12V) and with H-Ras(12V). Interestingly, this very same domain is also important for ErbB1/nucleolin interaction (24), suggesting that the nucleolin 212 C-terminal region is involved in the interactions of nucleolin with ErbB1 and Ras to enhance cell transformation and cancer development.

Nucleolin is a major nucleolar phosphoprotein of exponentially growing eukaryotic cells (14) and it is overexpressed in highly proliferating cells (44). Nucleolin was originally identified as a nucleolar protein, but is now considered to be a shuttle
protein present in the nucleolus, cytoplasm and at the cell surface (14, 17, 18). Interestingly it is also found on the cell surface of a wide range of cancer cells and is used as a diagnostic marker for cancer and for the development of anti-cancer drugs (18, 20, 44).

The ErbB subfamily of receptor tyrosine kinases has been implicated, more than other growth factor receptors, in the development of several human cancers (2). Elevated levels of the ErbB1 (EGFR), and/or its cognate ligands, have been identified as a common component of multiple cancer types and appear to promote tumor growth (45). Among the signaling pathways activated by the receptors is the prominent Ras/MAP kinase signaling cascade. Our study demonstrates a new link between nucleolin, ErbB1 and Ras proteins which may contribute to human cancer. Our results are consistent with a model whereby a crosstalk between the three proteins may contribute to human malignancies. Our model is based on several lines of evidence: i. ErbB1 pulls down nucleolin (12), ii. nucleolin pulls down both Ras and ErbB1, iii. Ras pulls down ErbB1 and nucleolin, iv. nucleolin, ErbB1 and Ras coimmunoprecipitate from lysates of the human cancer cell lines DLD1 and U87 that endogenously express all three proteins, and v. co-expression of nucleolin, ErbB1 and oncogenic Ras enhances cell transformation, as evidenced by anchorage-independent growth in soft agar and by tumor growth in nude mice.

Whereas the interactions between nucleolin and Ras are independent of the Ras nucleotide-binding-state, the interaction between nucleolin and ErbB1 is dependent on
the GDP/GTP state of Ras, as shown by the fact that the interaction is strongly increased by Ras-GTP compared to Ras-WT. These novel findings of cooperation between three oncogenes are clearly manifested in cell transformation and are highly relevant to human malignancies.

As discussed above, the ErbB receptors are involved in many human cancers and are therefore attractive candidates for targeted therapy. Two major classes of anti-ErbB therapeutics are already in clinical use or are in advanced development. The first is comprised of several Abs directed against the extracellular domain of ErbBs and the second of small-molecule tyrosine kinases inhibitors (TKIs) (11). Ras targeted therapy is at a less advanced stage. One class of putative Ras inhibitors is the farnesyltransferase inhibitors (FTIs) which have exhibited limited antitumor efficacy (46). A second class consists of farnesylcysteine mimetics including Salirasib (46) which showed significant efficacy in phase I/II trials (http://www.concordiapharma.com). Finally, nucleolin binds G-rich oligonucleotides (GROs) aptamers that induce antiproliferative responses. One of the aptamers named AS1411 (formerly AGRO100 or GRO26B-OH), is currently being tested as an anticancer agent in Phase II clinical trials (22). Using Salirasib and GRO to inhibit Ras and nucleolin respectively, we demonstrate that ErbB1 levels and EGF-mediated Erk activation are reduced. These observations further support the notion that nucleolin and Ras can affect receptor activities.

Our results demonstrating oncogenic cooperation between nucleolin, ErbB1 and Ras suggest that combined treatment may have stronger inhibitory effects than individual
inhibitors. Perhaps even more important is the finding that responsiveness to ErbB targeted therapy varies between patients, with no clear explanations for the basis of these differential responses. Interestingly, more recent experiments have shown that ~30% of colon cancer patients do not respond to ErbB1 inhibition, especially when they express mutated K-Ras (47, 48). It is not yet completely known why colon cancer patients harboring mutant K-Ras do not respond to Erbitux or Tarceva, although constitutive activation of downstream survival pathways is almost certainly one important contributor. It is tempting to also speculate that a robust nucleolin-mediated ErbB1 activation by mutant K-Ras may reduce or prevent the inhibitory effect of the drug. Whether or not this is the case, our results provide a strong rationale to consider a strategy of treatment of colon (or lung) cancer patients expressing mutant K-Ras with combinations of Ras and ErbB1 inhibitors, nucleolin and ErbB1 inhibitors or Ras and nucleolin inhibitors.

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Legends

Figure 1. Nucleolin interacts with Ras proteins. (A) HEK-293T cells were co-transfected with pEGFP-Ras isoforms and Flag-nucleolin expression vectors as indicated. As a control, cells were transfected with pEGFP-K-Ras(12V) alone. The resulting cell lysates were subjected to immunoprecipitation with anti-Flag monoclonal Abs and immunoblotted with anti-GFP polyclonal Abs and anti-Flag Abs. Total cell lysates are shown in the lower panels. (B) HEK-293T cells were co-transfected with N-Ras or N-Ras(13V) expression vectors as indicated. Cell lysates were subjected to immunoprecipitation with anti-nucleolin Abs and immunoblotted with anti-pan Ras or anti-nucleolin Abs as indicated. Total cell lysates are shown in the lower panels.

Figure 2. ErbB1-nucleolin interaction increases in the presence of activated Ras. HEK-293 cells stably expressing ErbB1 were transfected with expression vectors for each of the three Ras isoforms as indicated. The resulting cell lysates were subjected to immunoprecipitation with anti-nucleolin Abs and immunoblotted with anti-ErbB1 polyclonal or anti anti-pan Ras Abs (A,B). ErbB1 receptor precipitation by nucleolin Abs was increased 1.38 ± 0.03 fold, 1.57 ± 0.24 fold and 1.88 ± 0.23 fold in the presence of K-Ras(12V), H-Ras(12V) and N-Ras(13V) compared to their wild type forms respectively (n=3). (C). HEK-293 cells stably expressing ErbB1 were transfected with
pcDNA3 or with K-Ras(12V) expression vector. The resulting cell lysates were subjected to immunoprecipitation with anti-Ras Abs and immunoblotted with anti-ErbB1, anti-nucleolin or anti-Ras Abs as indicated. (D). ErbB1-Nucleolin-Ras co-immunoprecipitation in U87 and DLD1 cells. Lysates from DLD1 cells and U87 cells were subjected to immunoprecipitation with anti-nucleolin Abs and immunoblotted with anti-ErbB1, anti-nucleolin and anti-Ras Abs. As a control, lysates were precipitated with irrelevant (anti-GFP) Abs. The total input lysates are presented in the columns on the right.

Figure 3. The 212 C-terminal amino acids of nucleolin are important for nucleolin-Ras interaction. (A) Schematic presentation of nucleolin deletion mutants. (B-D) HEK-293T cells were co-transfected with expression vectors for Ras isoforms and pEGFP-nucleolin-Myc-tag mutant expression vectors as indicated. The resulting cell lysates were subjected to immunoprecipitation with anti-pan-Ras Abs and immunoblotted with anti-GFP or anti Ras Abs.

Figure 4. Cellular distribution of K-Ras (12V) or H-Ras (12V), nucleolin mutants and ErbB1. A. HEK-293T cells were transfected with GFP-K-Ras(12V) or H-Ras (12V) and either pDsRed, pDsRed-nucleolin, pDsRed-N-ter or pDsRed-212-C-ter as indicated. At 48 h following transfection, cells were fixed and subjected to confocal microscopy analysis. GFP-K-Ras(12v) or H-Ras (12V) are visualized in green and
pDsRed expression vectors are seen in red. Representative cells are presented. B. HEK-293T cells were transfected with GFP-K-Ras(12V), pCDNA3-ErbB1 and either pDsRed, pDsRed-nucleolin, pDsRed-N-ter or pDsRed-212-C-ter as indicated. At 48 h following transfection, cells were fixed and subjected to immunostaining with anti-ErbB-1 followed by Alexa fluor 405-labeled goat anti rabbit secondary antibodies. Confocal microscopy images of representative cells are shown. ErbB1 is visualized in blue, GFP-K-Ras(12v) in green and pDsRed expression vectors are seen in red.

**Figure 5. A. Activated Ras reduces ErbB1 disappearance following EGF stimulation.** Total cell lysates of HEK-293 cells stably expressing ErbB1 were transfected with GFP or Ras proteins, as indicated. 48 hr post transfection, cells were deprived of serum for 2 h and then treated with EGF (100 ng/ml) for the indicated times. Total cell lysates were analyzed by western blot, using anti-ErbB1 Abs. As a control, total cell lysates were immunoblotted with anti-tubulin Abs. Note that in the presence of activated Ras, ErbB1 is more stable following EGF stimulation. **B. Ras and nucleolin inhibition reduces receptor levels and Erk activation.** DLD1 cells were treated with FTS 75μM, GRO 5μM or both for 48 h, followed by EGF (100ng/ml) treatment for the indicated time periods. Total cell lysates were analyzed by western blot, using anti-ErbB1 and anti p-Erk Abs. As a control, total cell lysates were immunoblotted with anti-tubulin Abs. Note Gro and FTS treatments reduced the ErbB1 and phosphorylated Erk levels in response to EGF stimulation. **C. H-Ras (12V)**
enhance sensitivity to GRO mediated cell growth inhibition. NIH3T3 cells were stably transfected with pZIP empty vector or pZIP-H-Ras (V12) and expression of H-Ras was analyzed by Western blot. Stably transfected NIH3T3 cells were plated at low density in 96-well plates and incubated 18 hours at 37°C to allow adherence. Cells were then treated with 10 μM GRO or 10 μM CRO (control oligonucleotide) for four days. Proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (13). Data are presented as percentage of control untreated cells (mean±SE, n=3).

Figure 6. MDCK clones ectopically expressing pDsRed-Nucleolin, GFP-ErbB1 and H-Ras(12V) induce robust anchorage-independent growth. MDCK cells stably expressing pDsRed-Nucleolin alone, pDsRed-Nucleolin and GFP-ErbB1, GFP-H-Ras (12V) alone, pDsRed-Nucleolin and GFP-H-Ras or pDsRed-Nucleolin, H-Ras (12V) and GFP-ErbB1 were seeded into soft agar (1,000 cells/well in 96 well plates) in culture medium containing 10% FBS and 0.3% agar. The extent of colony formation was determined 8 days later. Cells were dyed with MTT and the wells were photographed and colonies were counted. (B) Results were quantified using the image analyzer program Image Pro-Plus. The results are presented as total number of colonies with size above 0.01 mm². Results are presented as percentage of the control untreated cells, and are the mean ± SD of three determinations (n=6). Each
experiment was repeated at least three times with similar results (***p< 0.001 and **p< 0.01 respectively).

**Figure 7. Nucleolin, ErB1 and H-Ras(12V) synergize in mediating tumor growth in nude mice.** The MDCK clones described for Figure 7 were grown, detached, washed with PBS and resuspended as detailed previously (30). On day zero, 2 × 10^6 cells of each clone in 0.1 ml of PBS were implanted subcutaneously just above the right femoral joint of nude CD1-Nu mice. Tumor volumes were then monitored on the indicated days (30). Each group of mice contained 6 mice and the mean tumor volumes ± SD are shown. Mock, nucleolin, nucleolin plus ErbB1 cells barely grew and are denoted with open circles, triangles and squares respectively. H-Ras(12V) (closed triangles) and H-Ras(12V) plus nucleolin (closed diamonds) grew small tumors. H-Ras(12V) plus nucleolin and ErbB1 (closed squares) grew large tumors. p< 0.05, p< 0.01 as compared to Mock controls.
References


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Figure 1
Figure 2
Figure 3

A. Nucleolin

B. K-Ras12V

C. H-Ras12V

D. N-Ras12V
Figure 5

A.

B.

C.

Figure 5
Figure 7

Tumor Growth
Oncogenic synergism between ErbB1, nucleolin and mutant Ras

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