MNK1 and MNK2 Regulation in HER2-overexpressing Breast Cancer Lines

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The functional role of MAP4 kinase-interacting kinase 1 (MNK1) and MNK2 in signal transduction is one of the major unsolved problems in signal transduction (1), and only recently has a MNK1 function been shown in translation of cytokines (2). Likewise, there are still large gaps in our understanding of how MNKs are regulated by MAP kinases. Human MNK1 was cloned as a novel substrate for ERK1, and the phosphorylation site identified was Thr-334 in the C terminus (3). MNK1 and MNK2 contain a canonical Thr-Pro motif for MAPKAP kinases in the T-loop, nine residues N-terminal to the conserved APE motif. Phosphorylation of the canonical Thr-Pro motif is required for activation of the RSK C-terminal domain (4), the MSK1 C-terminal domain (5), and MAPKAP kinase 2 (6). For these MAPKAP kinases, the canonical phosphorylation is the only phosphorylation required in the T-loop. However, MNK1 and MNK2 have atypical T-loops as the DFG motif is replaced by DFD, and in the only structures available (MNK2 (7)), the MNK T-loop has out-in conformations requiring a conformational switch to coordinate with Mg2+/ATP. In the T-loop of MNKs, there is a second Thr-Pro motif, which is conserved even in Drosophila melanogaster Mnk (7). The double alanine mutant of huMNK1 is inactive (T209A/T214A), and the T-loop is phosphorylated at both sites, as assessed by Thr to Ser mutations, peptide mapping, and phosphoamino acid analyses of resolved peptides (8). The available phosphospecific antibody (Cell Signaling) was made to a proprietary phosphopeptide containing both Thr-209/Thr-214 phospho-sites. The role of the canonical Thr-214 site, in particular, has not been deconvoluted and deserves more study. We investigated regulation of MNK phosphorylation with a phosphospecific antibody we generated for the canonical Thr-214-Pro as well as the available phospho-Mnk1 (Thr-197/202) antibody (Cell Signaling).

Human epidermal growth factor receptor 2 (HER2) (also known as ERBB2/Neu) is frequently overexpressed and/or mutated in aggressive breast cancers (9). HER2 is the molecular target of trastuzumab (HerceptinTM). Trastuzumab is initially effective, but loss of activity during prolonged treatment has been reported (10), an indication that additional therapeutic targets must be identified. We studied MNK1 and MNK2 and selected MAP kinase signaling pathways in a panel consisting of three cell lines that overexpress HER2 (HCC1419, AU565, and SKBR3), a cancer line that expresses HER2 (MCF7), and immortalized but nontumorigenic MCF10A cells for comparisons. The ERK pathway is altered in breast cancer cell lines that overexpress HER2, some by protein content and all by phosphorylation. MNK1 and MNK2 have increased and sustained activation in HER2-overexpressing cells, and treatment of AU565 cells with the MNK inhibitor CGP 57380 reduces colony formation in soft agar. This result identifies MNKs as therapeutic targets for breast cancers that have increased activation of the MNKs.

EXPERIMENTAL PROCEDURES

Reagents—Human cell lines MCF10A, MCF7, and HER2-overexpressing lines HCC1419, AU565, and SKBR3 were...
obtained from the American Type Culture Collection. The bicistronic plasmid for active ERK2 is from Melanie Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas) (12). The bicistronic vector for active p38 was described previously (6). The construct for *Escherichia coli* expression of His₆-human MNK1 was described (13); the construct for His₆-T334D MNK1 is the same except for the T334D mutation. Curt Hagedorn (Department of Internal Medicine, University of Kansas, Kansas City) generously provided eIF4E protein. Antibodies purchased from Cell Signaling Technology are as follows: dual phospho-Mnk1 (Thr-197/202) (catalog number 2111), phospho-eIF4E (Ser-209) (catalog number 9741), and active p38 (Thr-180/Tyr-182) (catalog number 9211); antibodies purchased from Santa Cruz Biotechnology are as follows: MNK1 C20 (catalog number 6965), MNK2 S20 (catalog number 6964), RSK1 C-21 (catalog number 231-G), RSK2 C-19 (catalog number 1430), and p38 N-20 (catalog number 728-G) all goat antibodies and mouse anti-ERK2 monoclonal antibody (catalog number 1647). The HER2 antibody (21N) (catalog number RB-103) was from Lab Vision Corp. FLAG antibody (M2) was from Sigma (catalog number F-3165). Phospho-ERK antibody was produced for us by Promega to a proprietary immunogen. Purified mouse anti-ERK1 monoclonal antibody (catalog number 554100) was from BD Biosciences, and the monoclonal actin antibody (catalog number 69100) was from MP Biochemicals, antibody to eIF4E, as described previously (6). The construct for p38MAPK, pELF4E, Thr(P)-214, and active p38, a minimum of 25 μg was used. For MNK1, MNK2, the dual phospho-MNK, pELF4E, Thr(P)-214, and active p38, a minimum of 2 μg, but frequently 5 μg, was used. To observe the HER2 band in MCF10A cells, 30 μg was used.

**Mutagenesis of MNK1**—The single mutations of MNK1 were created using site-directed mutagenesis, and the results were confirmed by DNA sequencing.

**Kinase Assays**—Kinase activity of the purified GST or His₆-tagged proteins were performed at 30 °C in 20-μl reactions containing (final concentrations) 25 mM Hepes, pH 7.4, 10 mM β-glycerophosphate, 2 mM dithiothreitol, 0.4 mg/ml bovine serum albumin, 50 μg/ml eIF4E, 15 μg/ml recombinant enzyme, 10 mM MgCl₂, 50 μM [γ⁻³²P]ATP (~4000 cpm/pmoul). Phosphate incorporation was quantified using a PhosphorImager and equal protein loading by Coomassie staining. For kinase assays of expressed proteins, cells were transfected with variants of FLAG-MNK variants, and after 24–30 h, the transfected cells were either left untreated or treated with 5% calf serum for 10 min or 50 μM sodium arsenite for 30 min, before harvesting in lysis buffer (300 μl). Resultant lysates were cleared and then immunoprecipitated with 1 μg of FLAG antibody, washed extensively, and subjected to in vitro kinase assays with eIF4E (0.5 μg per reaction) essentially as described previously (14).

**Colony Formation Assays**—Growth in soft agar of AU565 cells was assessed in 6-well plates. Cells (at 1 × 10⁶) were plated on top of a thin layer of media containing 0.7% agar in complete media with 0.45% agar and varying concentrations of CGP57380 or the highest amount of Me₂SO used. After 1 day, another layer of 0.7% agar media were placed on top, and every 3–4 days several drops of complete media were added to keep the plates moist. At 3 weeks the cells were stained with crystal violet (0.005%), counted, and photographed.

**RESULTS**

**MNK1 Activation by p38α or ERK2 MAP Kinases in *E. coli***—We reconstituted the MNK1 kinase cascade in *E. coli* (Fig. 1A) using a co-expression strategy. GST-MNK1 was co-expressed with bicistronic plasmids producing either active ERK2 or active p38α. Equal amounts of purified protein from the preparations were assayed for kinase activity in the presence or absence of eIF4E (Fig. 1A). Co-expression with active ERK2 or active p38α greatly increases GST-MNK1 phosphotransferase activity toward eIF4E (Fig. 1A, bottom panel); GST-MNK1 expressed...
 alone is not phosphorylated on the T-loop (Fig. 1B) and does not phosphorylate eIF4E (data not shown). Phosphorylation of eIF4E by active GST-MNK1 is not because of residual co-purified MAP kinases, demonstrated for p38α by inclusion of SB203580 in the assays (data not shown). The T334D mutant can be phosphorylated by p38α in vitro (data not shown). The T334D mutant did not phosphorylate eIF4E (data not shown). The T334D mutant was more effective in producing the active, phosphorylated MNK1 T334D proteins in parallel with the co-expressed phosphorylated proteins for eIF4E kinase activity using recombinant eIF4E and [γ-32P]ATP. The T334D mutation weakly increases autophosphorylation activity, but under our conditions, the T334D mutant did not phosphorylate eIF4E (data not shown). The T334D mutant can be phosphorylated in vitro at Thr-214 and appears to be a better substrate for p38 than the wild type enzyme (Fig. 1B). The T334D enzyme may require activating phosphorylations in the T-loop (Fig. 1E) by MAP kinases to display constitutive activity in cellular functional assays (15, 16), because the T334D mutation, by itself, does not appear to activate MNK1 in vitro.

Reconstitution of MNK1 activation in E. coli was also demonstrated by T-loop phosphorylation. Co-expression of GST-MNK1 with either active p38α or active ERK2 caused a “gel shift” of GST-MNK1 to a form of slower mobility indicative of dual phosphorylated MNK1 than the bicistronic plasmid for active ERK2. Anti-Thr(P)-214 recognizes both the upper and lower bands. This confirms that the antibody is specific for Thr-214 because recognition of both bands is lost with a T214A mutant but not the T209A mutant.

Phospho-Mnk1 (Thr-197/202)TM from Cell Signaling Technologies (herein called dual phospho-Mnk) was made to a proprietary immunogen peptide that was dually phosphorylated. We generated single threonine to alanine mutations for Thr-209 and Thr-214 in the T-loop of human GST-MNK1 to study MNK phosphorylation by either active ERK2 or p38α (Fig. 1D). GST-MNK1, expressed with either active ERK2 or p38α, generates a doublet detectable with phosphospecific antibodies to anti-Thr(P)-214. The bicistronic plasmid for active p38α was more effective in producing the active, phosphorylated MNK1/2 Breast Cancer phosphorylation (Fig. 1, A, C, and D). However, p38 was more effective at inducing the shift; possible explanations include better production of active p38 in the co-expression system or the presence of additional p38 sites on GST-MNK1. Incubation of GST-MNK1 or GST-MNK1 T334D with active p38α and ATP/Mg2+ in vitro induced Thr-214 phosphorylation in a time-dependent fashion (Fig. 1B). GST-MNK1 that had been co-expressed with active ERK2 was strongly reactive with anti-Thr(P)-214 antibody, whereas protein expressed alone was not (Fig. 1C). We conclude that the canonical Thr-Pro motif in the T-loop of MNK1 is phosphorylated by p38α MAP kinase or by ERK2, and the anti-Thr(P)-214 antibody is phosphospecific.

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Phosphorylation of Thr-209 and Thr-214 in MNK1 in MCF7 Cells—Wild type, T209A, and T214A MNK1 were expressed in MCF7 cells and subjected to Western blot analysis to correlate phosphorylations of the T-loop with activity (Fig. 2A). Transfection of wild type MNK1 increased phosphorylation of eIF4E, but addition of the single mutants did not detectably increase eIF4E phosphorylation. Anti-Thr(P)-214 gave a strong signal to wild type MNK1 as well as to the T209A mutant but did not recognize T214A MNK1, despite equivalent expression of the proteins. We conclude that the canonical Thr-Pro (Thr-214) is phosphorylated in MCF7 cells.

Dual phospho-Mnk antibody was reactive with FLAG-MNK1 expressed in MCF7 cells, and reactivity was greatly reduced by the T209A single mutation (Fig. 2A). The dual phospho-Mnk antibody from Cell Signaling is phosphospecific as expected and, in this experiment, largely dependent on phosphorylation of the noncanonical Thr-Pro motif site, Thr-209 in our numbering. Reactivity of MNK1 with dual phospho-Mnk was less affected by alanine substitution of Thr-214 compared with wild type MNK1 in in vivo experiments, but this difference in reactivity was not observed in the in vitro experiments (Fig. 1D). Because MNKs are the physiological eIF4E kinases (1), these results together prove the canonical Thr-Pro motif (Thr-214) is required but not sufficient for phosphorylation of eIF4E.

We confirmed the requirement for both Thr-209 and Thr-214 in experiments in which MCF7 cells were treated by addition of fresh serum or sodium arsenite (Fig. 2B). Sodium arsenite is a strong activator of stress-activated MAP kinases, including p38 and the agent stimulates eIF4E phosphorylation at Ser-209 (18, 19). Wild type MNK1 recovered from transfected MCF7 cells has eIF4E kinase activity that was modestly stimulated by additions of serum or sodium arsenite. No kinase activity was detectable for T209A or T214A mutants, despite similar expression and recovery. T209A and T214A mutants remain inactive despite additions of fresh serum or sodium arsenite to MCF7 cells. These independent results confirm that MNK1 enzymatic activity in cells requires phosphorylation at both the Thr-209 and Thr-214 sites.

Characterization of ERKs and Their RSK and MNK Targets in Breast Cancer Cell Lines—Human cell lines HCC1419, AU565, and SKBR3 greatly overexpress HER2, and MCF7 slightly overexpresses HER2 (Fig. 3A). MCF10A cells are immortalized but not tumorigenic (20), and they express HER2 at low levels. We characterized phosphorylation status of MNKs, as well as RSKs and MAP kinases in lysates prepared from equal numbers of cells collected although subconfluent and still growing in complete media. Lysates were analyzed for protein content and
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ERK1 and ERK2—A monoclonal antibody specific for ERK2 and unreactive with ERK1 revealed that ERK2 expression is higher in the HER2-overexpressing lines (HCC1419, AU565, and SKBR3) than in MCF7 and MCF10A (Fig. 3A). The same lysates from Fig. 3A were analyzed for MNK levels, activation status, and activity. A, levels of MNK1 and MNK2 do not vary considerably in the breast cancer lines. There is a cross-reacting band on the MNK2 Western blot (identified with *), and the upper bands of both doublets correspond with the bands observed with the phospho-specific antibodies. The bottom band of MNK1 is observed with the anti-Thr(P)-214 antibody. B, phospho-MNK antibodies Thr(P)-214 and dual phospho-MNK have different profiles in the breast cancer cells, and the Thr(P)-214 antibody recognizes an unknown protein in MCF10A cells, but for both antibodies MNKs are more phosphorylated in the HER2-overexpressing cell lines. Two more cross-reacting bands are observed, one with the dual phospho-MNK and the other with Thr(P)-214 only in MCF10A cells (both are identified with *). C, another set of lysates was also probed for Ser(P)-209 eIF4E, total eIF4E, dual phospho-MNK, and actin, and MNK activity indicated by phosphorylation of eIF4E is highest in the breast cancer lines. Note that phosphorylation of MNK2 and eIF4E was more variable in the MCF7 cell line. In some blots it was unobserved (A), and in others phosphorylation was easily observed (B). D, MCF10A with and without overexpressed HER2 was grown as described and Western blotted to determine whether HER2 status would alter the specific reactivity with the anti-Thr(P)-214 antibody; it did not impact observation of that band but did lower phosphorylation of p38 and increase phosphorylation of a RSK protein.

Phosphorylation of Ser-386 is a sensitive marker for the ERK-RSK pathway in breast cancer. Ser-386 lies in the linker between the RSK kinase domains (22), and it is conserved in RSK1–4 (14) and is phosphorylated intramolecularly by the C-terminal kinase. Phosphorylation of RSK1–4 was increased in all the breast cancer lines in comparison with MCF10A cells (Fig. 3A).

MNK1—Anti-MNK1 (C20) (see under “Reagents”) detected two closely spaced bands of ~46 and ~48 kDa in all the lines. MNK1 has alternative transcripts. Isoform 2 (e.g. chorioncarcinosoma cDNA BC002755) encodes 465 amino acids with a predicted mass of 53.3 kDa and uses exon 9. We did not find isoform 2 in the huEST data base by searching with exon 9, using appropriate methods for short sequences. This suggests isoform 2 is rare. Isoform 1 (accession number AB000409), as cloned from HeLa cells by Fukunagawa and Hunter (3), lacks exon 9 and encodes 424 amino acids, with a predicted mass 47.4 kDa. MNK1b (17), encoded by an alternative cDNA from HL-60 leukemia cells, lacks the C terminus and would not be recognized by anti-MNK1 (C20). Anti-MNK1 (C20) detects bands of the apparent mass for phosphoforms of MNK1a isoform 2, and in agreement with Myc-tagged MNK1a isoform 2 (17), the doublet of MNK1 we detect runs just beneath a 50-kDa marker on SDS-PAGE (Fig. 4A). The doublet did not collapse upon treatment with phosphatase inhibitors (data not shown). MNK1 levels are not appreciably different between the breast cancer lines.

MNK2—MNK2 was identified through yeast two-hybrid interaction with estrogen receptor β (23). This interaction has not been confirmed or extended, so the significance is unclear.

FIGURE 4. The activation of MNKs correlates with breast cancer. The same lysates from Fig. 3A were analyzed for MNK levels, activation status, and activity. A, levels of MNK1 and MNK2 do not vary considerably in the breast cancer lines. There is a cross-reacting band on the MNK2 Western blot (identified with *), and the upper bands of both doublets correspond with the bands observed with the phospho-specific antibodies. The bottom band of MNK1 is observed with the anti-Thr(P)-214 antibody. B, phospho-MNK antibodies Thr(P)-214 and dual phospho-MNK have different profiles in the breast cancer cells, and the Thr(P)-214 antibody recognizes an unknown protein in MCF10A cells, but for both antibodies MNKs are more phosphorylated in the HER2-overexpressing cell lines. Two more cross-reacting bands are observed, one with the dual phospho-MNK and the other with Thr(P)-214 only in MCF10A cells (both are identified with *). C, another set of lysates was also probed for Ser(P)-209 eIF4E, total eIF4E, dual phospho-MNK, and actin, and MNK activity indicated by phosphorylation of eIF4E is highest in the breast cancer lines. Note that phosphorylation of MNK2 and eIF4E was more variable in the MCF7 cell line. In some blots it was unobserved (A), and in others phosphorylation was easily observed (B). D, MCF10A with and without overexpressed HER2 was grown as described and Western blotted to determine whether HER2 status would alter the specific reactivity with the anti-Thr(P)-214 antibody; it did not impact observation of that band but did lower phosphorylation of p38 and increase phosphorylation of a RSK protein.

equalized in sample buffer (see under “Experimental Procedures”). Lysates were stored frozen at −70 °C to allow comparisons of multiple signaling components by Western blotting. Equal protein loading was confirmed by Western blotting for actin.

ERK1 and ERK2—A monoclonal antibody specific for ERK2 and unreactive with ERK1 revealed that ERK2 expression is higher in the HER2-overexpressing lines (HCC1419, AU565, and SKBR3) than in MCF7 and MCF10A (Fig. 3A). An ERK1 monoclonal antibody shows ERK1 is not significantly up-regulated in the HER2-overexpressing lines in comparison with MCF7, and surprisingly ERK1 is not detected in the MCF10A cells. This was a striking, consistent difference in expression for ERK1 in MCF10A versus all the other lines examined.

ERK2 activity, inferred from reactivity to phospho-ERK antibody that detects dually phosphorylated kinase, was much higher in the HER2-overexpressing lines in comparison with MCF7 and MCF10A cells, as reported for HER2-overexpressing lines previously. There was active ERK2 (but not ERK1) in proliferating MCF10A cells but not detectably in MCF7. This is a relative absence in MCF7, as active ERK1 and ERK2 are detectable in MCF7 with longer exposures and/or by increasing the amount of total lysate protein.

MAP Kinase p38α—The MAP kinase p38α was most active in MCF10A cells in comparison with the lines from human breast cancer (Fig. 3A). Total p38α MAP kinase was expressed at similar levels. The p38α MAP kinase pathway is activated by cellular stress, which we tried to minimize by comparisons of subconfluent cells in complete media. Activation of p38α correlates with differentiation and cell cycle exit in the intestinal epithelium (21).

RSKs—Specificity of antibodies to RSK1 and RSK2 was established in control experiments (Fig. 3B). HA-RSK1 and HA-RSK2 were expressed in BHK21 cells and immunopurified; equivalent loading for Western blots was verified with the HA tag. RSK1 C-21 (goat) has much higher reactivity with HA-RSK1 than HA-RSK2 and is thus relatively specific for RSK1. RSK2 C-19 (goat) detects HA-RSK1 and HA-RSK2 equally well. RSK, most likely RSK1, was increased in HER2-expressing lines (Fig. 3A). RSK2 expression was variable, using RSK2 C-19, and expression correlated with active ERK2.

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Human MNK2 has two reported alternative transcripts, encoding MNK2a (465 amino acids) and MNK2b (414 amino acids), with predicted 52- and 46.7-kDa masses. Anti-MNK2 (S20) detects two doublets of major bands, one at ~52–55 kDa and one more closely spaced at ~72–75 kDa. The ~52–55-kDa doublet is compatible with MNK2a (Fig. 4A). Both the dual phospho-MNK and anti-Thr(P)-214 antibodies detect the 72–75-kDa bands with longer exposures (Fig. 4B). This suggests breast cancer cells express either an uncharacterized form of MNK2 or a cross-reacting MNK2-like protein. The MNK2 bands also do not vary considerably between the breast cancer lines, although the AU565 cells have slightly less of the ~52–55-kDa doublet (Fig. 4B).

**MNKs Are Most Active in HER2-overexpressing Lines**—Dual phospho-Mnk antibody specifically detects active MNKs. Dual phospho-Mnk antibody has the strongest reactivity with the upper, slower migrating band of MNK1a, the upper band of the MNK2a doublet at ~52–55 kDa, and the upper band of the MNK2-like doublet at ~72–75 kDa. MNK1a was most active in the HER2-overexpressing lines in the order SKBR3 = AU565 > HCC1419, determined by different exposures to film (Fig. 4, B and C). Likewise, MNK2a was most active in the same lines, with the same relative order. Dual phospho-Mnk antibody reactivity to MNK2a matched the upper band of the doublet in overlays of film and blot. Our results establish that MNK2 phosphorylation in breast cancer cells is neither uniform nor constitutive under conditions of our analyses and may relate to their biology. This is different from other cell lines that have high constitutive activity of MNK2 (24). Reactivity of the cross-reacting MNK2 bands at ~72–75 kDa was more variable and did not correlate with HER2 overexpression.

**Phosphorylation of eIF4E in HER2-overexpressing Lines**—Phosphorylation of eIF4E assayed with phosphospecific antibody for Ser-209 was highest in HER2-overexpressing lines and correlated with dually phosphorylated MNK1a and MNK2a and not with the cross-reactive MNK2-like protein running at ~72–75 kDa (Fig. 4C). Total levels of eIF4E were relatively equivalent between the breast cancer cell lines, but there is less eIF4E in the MCF10A cells.

**Anti-Thr(P)-214 Detects Dually Phosphorylated MNKs but Is Selective for Singly Phosphorylated MNK**—Anti-Thr(P)-214 is reactive with both the upper and lower phosphoforms of endogenous MNK1a (Fig. 4B). Anti-Thr(P)-214 is strongly reactive with the lower band of the doublet even in MCF10A cells, suggesting MNK1 Thr-214 is phosphorylated basally in cells, and activation and the gel shift are because of additional phosphorylation(s), presumably at Thr-209. Recognition of the upper band by anti-Thr(P)-214 becomes obvious in overexposures (Fig. 5 and data not shown). Additionally, in the bacterial expression system (Fig. 1D), we observe that the Thr(P)-214 antibody detects the phosphorylated T209A protein better than the wild type MNK, which has the highest amount of dually phosphorylated protein. These results suggest additional phosphorylation at Thr-209 in the T-loop decreases recognition by the anti-Thr(P)-214 antibody that was made as a single site phosphospecific antibody.

**Anti-Thr(P)-214 Detects an Unidentified MCF10A-specific Protein**—An unidentified MCF10A protein has prominent reactivity with anti-Thr(P)-214 (Fig. 4B). This band at ~69 kDa does not match the mobility of the principal MNK bands and was not recognized by the dual phospho-MNk antibody, indicating that if it is a form of MNK then it is inactive. This prominent band was not down-regulated in ERBB2-transformed MCF10A cells, although phosphorylation of p38 was decreased and phosphorylation of an RSK protein was increased (Fig. 4D) (cells were a gift of Dr. Sam Lee, Harvard University). Attempts to identify the prominent band were unsuccessful.

**ERKs, RSKs, and MNKs Are Constitutively Active in AU565 Cells**—AU565 was selected for comparison with MCF7 and MCF10A in a time course experiment, and cells were serum-starved for 5 h and then treated with 5% serum (Fig. 5). ERK1 and ERK2 were phosphorylated constitutively in AU565, and phosphorylation was not down-regulated by serum starvation. In MCF7 cells, serum induced only a transient phosphorylation of ERK1 and ERK2. In MCF10A cells, ERK2 was not detectably phosphorylated after serum starvation, and serum re-addition induced sustained ERK2 phosphorylation in comparison with MCF7. RSK protein kinase(s) were phosphorylated constitutively at Ser-386 in AU565 and at much higher levels than in MCF7 or MCF10A. Anti-Ser(p)-386 also detected a cross-reactive 66-kDa phosphoprotein that was absent in serum-starved AU565 and MCF7 cells and appeared rapidly after serum addition. Phosphorylation of this unidentified protein was sustained in AU565 and was transitory in MCF7 cells. MNK1 was activated by readdition of serum in AU565 cells, indicated by the appearance of the upper MNK1 band detectable with anti-Thr(P)-214 that correlates with the dually phosphorylated active form (Fig. 5, light exposure). In MCF10A cells, we observed time-dependent phosphorylation of MNK1 because MNK1 but not MNK2 runs sufficiently below the interfering MCF-10A-specific band. By using the Thr(P)-214 antibody, MNKs are constitutively phosphorylated in AU565 cells in comparison with MCF7 when starved of serum, but dual phosphorylation of MNK1 is induced by serum (appearance of the
DISCUSSION

MNK1 and MNK2 Regulation by Phosphorylation—We established that MNK1 like MNK2 (24) requires Thr-209 and Thr-214. Phosphorylation of MNK1 at the canonical Thr-Pro motif (Thr-214) is required but not sufficient for MNK1 to be active as an eIF4E kinase. The phosphorylation of these sites in the T-loop requires an active p38α or ERK protein kinase, and either MAP kinase can induce phosphorylation of both sites in vitro or in E. coli reconstitution assays. Phosphorylation of the T-loop may be ordered. Anti-Thr(P)-214 detects a band of slower mobility that is not dually phosphorylated. This suggests that Thr-214 may be basally phosphorylated in growing cells. The dually phosphorylated MNK1 detected with dual phospho-Mnk antibody is shifted to slower mobility with respect to this band but is still detectable with anti-Thr(P)-214 with longer exposures.

Phosphorylation at Thr-209 may decrease reactivity for anti-Thr(P)-214 because the sites are close in primary sequence. This may be rationalized by the design of the two antibodies. The immunogen for anti-Thr(P)-214 necessarily was to a single phosho-site. Dual phospho-MNK (phospho-Mnk1-Thr-197/202) antibody was produced to a proprietary, dually phosphorylated immunogen.

The Thr-334 site in MNK1 was identified in vitro for ERK1 (3). Threonine to serine substitution with peptide mapping and phosphoamino acid analyses did not reveal any phosphorylation of Thr-334 (8). Nevertheless, replacement of this residue with aspartate (T334D) is an activating mutation for MNK1 in cells, revealed by functional assays (15, 16). The mechanism is unclear. Our assays of recombinant His6-MNK1 T334D showed that this mutant protein is not significantly phosphorylated in the T-loop and is minimally activated. The T334D mutant may be a better p38 substrate when compared with the WT-MNK1 (Fig. 1B). Thr-334 lies in a loop or turn, just following the α-helix at the end of the catalytic domain. In MAPKAP kinase 2 of the same family as MNK, a corresponding residue (Thr-317 to Pro) is phosphorylated by p38α, regulates nuclear export (26), and contributes to enzymatic activation (27). Acidic substitution at Thr-317 partially activates recombinant MAPKAP kinase 2. The AGC protein kinases have a similar site of phosphorylation in the turn loop, and in the case of protein kinase C the site is autophosphorylated and controls interaction with Hsp70 (28). A similar residue is phosphorylated in MNK2 but is not required for kinase activity (24). These precedents suggest the constitutive activity of MNK1 T334D observable in cells may result from a change in stability or cellular localization. An alternative explanation is that T334D is more readily phosphorylated in the T-loop or is a poor substrate for the MNK phosphatase.

ERKs and RSKs in Breast Cancer—Our findings point to distinct functions for ERK2 and ERK1. We had expected that ERKs would be strongly and uniformly active in all the lines overexpressing HER2. We found instead that ERK1 and ERK2 are distinctly regulated. ERK2 activation more closely parallels HER2 overexpression (Fig. 3A). ERK1 protein is not detectable in MCF10A cells. Remarkably, relatively little is known about the promoters of the ERKs or RSKs given clear evidence for overexpression of ERK2 in breast cancers (29). A correlation of active ERK2 with RSK2 expression (Fig. 7) should motivate studies of the role of upstream ERKs in RSK gene transcription or protein stability.
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Our studies reveal that MAP kinase pathways are dysregulated in HER2-overexpressing lines in comparison with MCF7 cells. MCF7 cells may not be representative of the most aggressive breast cancers. MCF7 cells arrest in G1 by a MEK1- and/or MEK2-dependent pathway activated by phorbol esters with induction of p21Cip/Waf1. MCF7 cells engineered to stably express colony-stimulating factor 1 (CSF-1) receptor also arrest in G1 when treated with CSF-1 by induction of p21Cip/Waf1 and by a PD98059 inhibitable mechanism (30).

SKBR3 cells in particular had low levels of active ERKs. MDA-MB231 cells, having highly active ERKs, are sensitized by MEK inhibitor U0126, whereas SKBR3 cells are not (31). SKBR3 cells have wild type PTEN and are inhibited by Herceptin\textsuperscript{TM}, whereas SKBR3 resistant to Herceptin\textsuperscript{TM} have lost PTEN expression (32, 33). The loss of PTEN causes hyperactivation of phosphatidylinositol 3,4,5-phosphate signaling. This suggests that a combination of rapamycin or an AKT inhibitor and Herceptin\textsuperscript{TM} might be more effective than Herceptin\textsuperscript{TM} alone.

The lack of expression of ERK1 protein in MCF10A cells may prove useful for investigation of ERK1- and ERK2-specific functions. Loss of ERK1 is unusual and suggests loss of ERK1 may be related to immortalization of these cells. A recent study shows that ERK1 protein may act as a negative regulator of growth by antagonizing ERK2 (34). None of the described cyrogenetic changes in MCF10A cells involve the ERK1 locus (20).

**MNKs in Breast Cancer**—It was surprising to us, given the many reports on elf4E in breast cancer (35, 36), that there have been no studies of MNKs in breast cancer. The hypothesis that MNK functions in mRNA translation has been obvious because elf4E was first established as a substrate (37), and MNK1 was shown to interact with the translational scaffold elf4G (8, 38). MNK1 phosphorylates elf4E at conserved Ser-209 but has other substrates. Loss of function of *Drosophila* MNK decreases viability, slows development, and decreases adult size. This is due to elf4E phosphorylation because overexpression of *Drosophila* MNK rescues lethality of elf4E hypomorphs dependent on intact Ser-209 (39).

elf4E is the ubiquitous binding protein for capped mRNAs, and it promotes stability and translation of mRNA. Cap-dependent translation is regulated by sequestration of elf4E as a complex with de-phosphorylated elf4E-binding protein. A role for Ser-209 phosphorylation in cap-dependent translation is based on an increase of affinity of phospho-elf4E for binding to the cap. A requirement for MNK1 in cap-dependent translation became less viable when elf4E Ser209A mutants had no effect on translation, and inviable when mice lacking both MNKs were overtly normal and elf4E phosphorylation was absent (1). This suggests elf4E phosphorylation has another function and that mammalian MNKs function in pathways that promote adaptation or survival.

The other function of elf4E is a nuclear function, principally shown from the work of the Borden laboratory (see Ref. 40). Overexpression of elf4E can transform NIH3T3 cells, enabling foci formation in soft agar, and transformation is decreased by S209A or S209D mutants (41). This result does not by itself prove elf4E phosphorylation is required for transformation, but it offers support. Importantly, inhibition of elf4E phosphorylation with CGP57380 decreases cellular content of cyclin D1 (42). One proposed mechanism is altered distribution of cyclin D1 mRNA (41). An element in the 3′-UTR of cyclin D1 was required for the positive effects of elf4E on transport of cyclin D1 mRNA to the cytoplasm, and expression of S209A/S201A mutants of elf4E increased the fraction of cyclin D1 in the nuclear compartment, implying phosphorylation is required for transport. These results suggest a role for MNKs in mRNA trafficking. Phosphorylation of elf4E by MNKs in breast cancer may selectively up-regulate cyclin D1 expression. The nuclear fraction of cyclin D1 localizes to PML bodies, and MNKs shuttle between the nucleus and the cytoplasm (43), and could phosphorylate the nuclear pool of elf4E. MNKs also co-localize with PML bodies (44).

MNKs appear required for translation of TNFα and certain other cytokine mRNAs (2, 11). The mechanism is seminally important but unclear. Heterogeneous ribonucleoproteins (hnRNP) are a family of proteins that are alternatively spliced and bind specific elements in the 3′-UTR of specific mRNAs to regulate them in several ways, e.g. splicing, nucleocytoplasmic trafficking, and translation. MNK1 phosphorylates hnRNP A1, an hnRNP that binds TNFα and other mRNAs (2). hnRNP A1 is up-regulated in some cancers (45), and silencing of hnRNP A1 promotes cell death of transformed but not normal cells (46). hnRNP proteins themselves are translationally controlled to alter abundance during the cell cycle (47). In *Drosophila*, translation of Oskar mRNA is repressed by binding an hnRNP A/B ortholog until the mRNA is properly localized. This may be a clue to the mechanism for MNKs and the role in TNFα mRNA, because inhibition of MNKs increased binding of hnRNP A1 to TNFα 3′-UTR. Phosphorylation of hnRNP A1 by MNKs may decrease binding (2) allowing specific subsets of mRNAs (e.g. TNFα) to be translated.

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**FIGURE 7.** Comparison of MAPKs and MNKs in MCF7 and other breast cancer cell lines. Affymetrix MAS 5.0 data for MCF7, ZR75, HS578T, and MDA-MB231 were downloaded from the Genomics Institute of the Novartis Research Foundation. The data for each indicated were transposed to a single Excel file and normalized relative to the highest expressing line. NCI-ADR-RES and MDA-MB435 misidentified as breast cancer lines were not included (58). Useful descriptions of their biology are found online in the breast cancer cell database at the University of Texas MD Anderson Cancer Center. Gene (probe number): RSK1 (203379); RSK2 (204906); MEK3 (215499); MEK6 (205698); p38α (202530); MEK1 (202670); MEK2 (202424); ERK1 (212046); ERK2 (212271); MNK1 (209467); and MNK2 (218205).
The specificity of MNKs for translation of specific mRNAs may also result from regulation at the 5′ end of the mRNA to promote alternative modes of translation by internal ribosomal entry sites (IRES). Expression of MNK1 T334D decreased cap-dependent translation relative to the IRES-driven cistron in a bicistronic reporter, and it was not possible to isolate stable lines that express constitutively active T334D (15). The ability of MNK to inhibit general protein synthesis to favor translation of a specific message was demonstrated in *Aplysia* for the mRNA for egg-laying hormone (48, 49). Several mRNAs encoding proteins important in cancer have an IRES, including cyclin D1, p27kip1, c-Myc (50), and IGF-1 receptor (51). MNK1 is a target of fusion transcription factors that result in acute myeloid leukemia. Three different fusion proteins, including PML-RARA, induce MNK1 protein by post-transcriptional stabilization of the protein despite repressing MNK1 mRNA. Inhibition of MNK1 by expression of a dominant-negative mutant (T209A/T214A) inhibits growth of leukemic cells, and inhibition of MNKs with CGP57380 enhanced differentiation responses (25), suggesting MNK1 is required for maintenance of this cancer.

Expression of MNKs and Their Activators in Other Breast Cancer Cell Lines—We compared expression of MNK1 and MNK2 and selected other genes, using data from the Genomics Institute of the Novartis Research Foundation, for MCF7, ZR75, HS578T, and MDA-MB231 (Fig. 7). MNK1 expression was highest in MDA-MB231 cells, a highly invasive line with constitutive ERK2 activation (31) that does not overexpress HER2 (52). MDA-MB231 cells also have the highest expression of MEK1, RSK1, MEK3, and p38α compared with the other lines. Of the potential MNK1 activators, expression of MEK3 and p38α appears most similar to MNK1. MNK2 expression was highly induced in ZR75, ZR75 cells overexpress HER2 (52) and are ER+ but tamoxifen-resistant (53). Overexpression of MNKs, particularly MNK2, may become important in breast cancer cell lines for survival and proliferation.

ERK1 expression is highest in MCF7 and much lower in the other lines. ERK2 expression is especially high in ZR75 relative to the other lines. These data are consistent with ERK1 and ERK2 having distinct functions. ERK2 and RSK2 have similar profiles, suggesting ERK2 may regulate expression of RSK2 or share regulation with RSK2.

**MNK1/2 Inhibition in HER2-overexpressing Cells Reduces Colony-forming Ability**—MNK1 and MNK2 activities are up-regulated in the HER2-expressing cell lines HCC1419, AU565, and SKBR3, assessed by the Thr(P)-214, dual phospho-Mnk antibodies, and phosphorylation of eIF4E. Other breast cancer lines MDA-MB231 and ZR75 have increased levels of MNK1 and MNK2 mRNA, which may lead to increased activation of the protein as well (Fig. 7), as expression of transfected WT-MNK1 enhanced phosphorylation of eIF4E in MCF7 cells (Fig. 2A). Increased activation of kinases because of HER2 overexpression has led to the development of many inhibitors in the pathway, targets that include HER1–4, Ras, Raf, MEK1/2, and more recently RSK2 (54, 55). It is known that dominant-negative MEK1 or the MEK1/2 inhibitor U0126 can inhibit colony formation of HER2/3-overexpressing cells (56) and that decreased expression of eIF4E protein (antisense) or the inability of eIF4E to be phosphorylated negatively impacts transformation of cells (41, 57). With this knowledge we tested the ability of CGP57380, a specific inhibitor of MNK1/2, to prevent colony formation of AU565 cells. The observed reduction of colony formation in the presence of CGP57380 (Fig. 6) suggests that MNKs may have a role in proliferation or invasiveness of breast cancers. In other work we have found that loss of the MNK protein or activity sensitizes fibroblast cells to serum withdrawal-induced apoptosis. A reasonable hypothesis would be that loss of MNK1/2 activity in the cancer cells sensitizes them to apoptosis. Cancers may acquire dependence on protein kinases that are not required for normal growth, and kinases, like MNKs, that are unnecessary for normal growth may be the best cancer targets (1). Our work should motivate studies of CGP57380 as a candidate therapeutic and MNKs as a potential drug target in breast cancer.

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