Tyrosine Kinase p56\textsuperscript{\textit{Lck}} Regulates Cell Motility and Nuclear Factor \(\kappa\)B-mediated Secretion of Urokinase Type Plasminogen Activator through Tyrosine Phosphorylation of I\(\kappa\)B\(\alpha\) following Hypoxia/Reoxygenation* 

Nuclear factor \(\kappa\)B (NF-\(\kappa\)B) plays major role in regulating cellular responses as a result of environmental injuries. The molecular mechanism(s) by which hypoxia/reoxygenation (H/R) regulates p56\textsuperscript{\textit{Lck}}-dependent activation of NF-\(\kappa\)B through tyrosine phosphorylation of I\(\kappa\)B\(\alpha\) and modulates the expression of downstream genes that are involved in cell migration in human breast cancer cells are not well defined. In this paper, we investigated the involvement of protein-tyrosine kinase p56\textsuperscript{\textit{Lck}} in the redox-regulated activation of NF-\(\kappa\)B following H/R in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. We demonstrated that H/R induces tyrosine phosphorylation of p56\textsuperscript{\textit{Lck}}, nuclear translocation of NF-\(\kappa\)B, NF-\(\kappa\)B-DNA binding, and transactivation of NF-\(\kappa\)B through tyrosine phosphorylation of I\(\kappa\)B\(\alpha\). Transfection of these cells with wild type Lck but not with mutant Lck F394 followed by H/R induces the tyrosine phosphorylation of inhibitor of nuclear factor \(\kappa\)B (I\(\kappa\)B\(\alpha\)) and transcriptional activation of NF-\(\kappa\)B, and these are inhibited by Lck inhibitors. 

In vitro kinase assay demonstrated that immunoprecipitated p56\textsuperscript{\textit{Lck}} but not Lyn or Fyn directly phosphorylate I\(\kappa\)B\(\alpha\) in presence of H/R. Pervanadate, H\(_2\)O\(_2\), and H/R induce the interaction between Lck and tyrosine-phosphorylated I\(\kappa\)B\(\alpha\), and this interaction is inhibited by Src homology 2 domain inhibitory peptide, suggesting that tyrosine-phosphorylated I\(\kappa\)B\(\alpha\) interacts with Src homology 2 domain of Lck. Luciferase reporter gene assay indicated that Lck induces NF-\(\kappa\)B-dependent urokinase type plasminogen activator (uPA) promoter activity in presence of H/R. Furthermore, H/R stimulates the cell motility through secretion of uPA. To our knowledge, this is the first report that p56\textsuperscript{\textit{Lck}} in presence of H/R regulates NF-\(\kappa\)B activation, uPA secretion, and cell motility through tyrosine phosphorylation of I\(\kappa\)B\(\alpha\) and further demonstrates an important redox-regulated pathway for NF-\(\kappa\)B activation following H/R injury that is independent of I\(\kappa\)B kinase/I\(\kappa\)B\(\alpha\)-mediated signaling pathways.

Tumor invasion, malignant progression, and distant metastasis depend on complex multistep processes. One prerequisite is the ability of tumor cells to initiate extracellular proteolysis, which is required for the crossing of tissue barriers, cell migration, extracellular matrix invasion, tissue remodeling, and angiogenesis. There is abundant experimental evidence that urokinase type plasminogen activator (uPA),\(^\text{1}\) a member of serine protease plays a major role in malignant progression and tumor metastasis (1). Up-regulations of uPA and urokinase type plasminogen activator receptor (uPAR) have been described in many human tumors. High levels of uPA and uPAR in tumor tissues are associated with poor prognosis of patients with cancer of the breast, lung, head and neck, uterine, cervix, bladder, and ovary (2). Most tumors are characterized by low extracellular pH, glucose depletion, high lactate levels, and regions with low oxygen tensions (3, 4). Low oxygen tension in tumors have been associated with poor outcome (5, 6), enhanced local or locoregional spread (7), and enhanced metastatic potential (8, 9). Hypoxia is a key parameter, able to modulate the expression of a variety of genes that are involved in tumor angiogenesis, malignant progression, and distant metastasis (10–12).

Lck, a member of the Src family non-receptor protein-tyrosine kinase, is mostly expressed in T cells and some B cells. Lck is also expressed in breast cancer tissues and cell lines (13, 14). Lck binds to the cytoplasmic domain of CD4 and CD8 and plays an essential role in T cell activation and development (15, 16). Lck is required for T cell receptor signaling in human Jurkat T cells and for antigen receptor-dependent cytolytic effector function in the CTTL-2 T cells (17, 18). Moreover, mice lacking expression of functional Lck or overexpressing an inactive form of Lck have severely disrupted thymocyte development (19, 20). Lck is a typical Src-like tyrosine kinase, and its activity is regulated by phosphorylation of a highly conserved tyrosine residue, Tyr-505, located near the carboxyl terminus (21, 22). The protein-tyrosine kinase C-terminal Src kinase phosphorylates Lck and is a natural inhibitor of Lck kinase activity (23). In vitro, Lck undergoes auto-phosphorylation at Tyr-394 (24, 25), and the extent of phosphorylation at Tyr-394 correlates with Lck activity and appears to be required for

---

* This work was supported in part by grants from the Department of Biotechnology (to the National Centre for Cell Science) of the Government of India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the Council of Scientific and Industrial Research, Government of India.

‡ To whom correspondence should be addressed. Tel.: 91-20-56909931 (ext. 203); Fax: 91-20-5692259; E-mail: gopalkundu@hotmail.com.

\(^1\) The abbreviations and trivial names used are: uPA, urokinase type plasminogen activator; uPAR, urokinase type plasminogen activator receptor; Lck, lymphocyte-specific protein-tyrosine kinase; H/R, hypoxia/reoxygenation; FITC, fluorescein isothiocyanate; NF-\(\kappa\)B, nuclear factor \(\kappa\)B; I\(\kappa\)B\(\alpha\), inhibitor of nuclear factor \(\kappa\)B; Luc, luciferase; pV, pervanadate; IKK, I\(\kappa\)B kinase; TNF\(\alpha\), tumor necrosis factor \(\alpha\); EMSA, electrophoretic mobility shift assay; FMA, phorbol 12-myristate 13-acetate; pp2, 4-amino-5-(4-chlorophenyl)-7-tert-butyl)pyrazolo[3,4-d]pyrimidine; emodin, 6-methyl-1,3,8-trihydroxyanthraquinone; TP, tyrosine phosphatase; SH2, Src homology 2.
maximum catalytic activity (26, 27). Studies of oxidative stress on Src family tyrosine kinases have yielded contradictory results. Nakamura et al. (28) reported that treatment of human peripheral blood T lymphocytes with oxidant diamide (1,1'-azobis(N,N-dimethyl formamide)) increased p56<sup>lck</sup> catalytic activity and induced phosphorylation of p56<sup>lck</sup> at both Tyr-394 and Tyr-505 residues (28). In contrast, H<sub>2</sub>O<sub>2</sub> increased the catalytic activity of Lck in most of the cell types (29) and phosphorylation of Tyr-394 is apparently required for H<sub>2</sub>O<sub>2</sub>-induced activation of Lck (30).

The NFκB family consists of several members including p65, p50, RelB, and c-Rel molecules (31, 32). NFκB is a redox-sensitive transcription factor usually retained in the cytoplasm by its inhibitory protein termed IκBα (33-35). The phosphorylation-dependent inactivation of IκBα leads to the translocation of NFκB into the nucleus, where it acts as a transcription factor. The phosphorylation of IκBα has been extensively studied, which results in establishment of two distinct pathways; one involves serine-threonine phosphorylation and degradation of IκBα through ubiquitination, and the other parallel and less explored pathway is through tyrosine phosphorylation of IκBα (36, 37). As compared with IKK-mediated serine phosphorylation of IκBα, tyrosine phosphorylation of IκBα is capable of activating NFκB in the absence of proteasome-dependent degradation of IκBα and the exact molecular mechanism(s) of activation of NFκB through tyrosine phosphorylation of IκBα are not well established.

The Src family tyrosine kinase p56<sup>lck</sup> is known to induce IκBα tyrosine phosphorylation and NFκB transactivation in T-lymphocyte upon pervanadate treatment (a tyrosine phosphatase inhibitor) (37, 38). Loss of tyrosine kinase p56<sup>lck</sup> in Jurkat cells abolished NFκB activation and partially suppressed and delayed phosphorylation of Tyr-42 of IκBα upon pervanadate treatment (38). Similarly, tyrosine phosphorylation of IκBα is observed in bone marrow macrophage following by TNFα treatment and this phosphorylation requires c-Src activity (39). Recent data demonstrate the involvement of c-Src kinase in H/R-induced tyrosine phosphorylation of IκBα. In this study, the triple knockout cell lines (c-Src<sup>−</sup>/H9251, Fyn<sup>−</sup>/H9251, and Yes<sup>−</sup>/H9251) show residual IκBα tyrosine phosphorylation upon treatment with H<sub>2</sub>O<sub>2</sub> or induction with H/R (40). These data clearly suggest that other tyrosine kinases are involved in these processes. Because most of the studies on IκBα tyrosine phosphorylation has been carried out in hematopoietically derived T-cells, the functional significance of these on breast cancer-specific epithelial models remains unsolved.

uPA is a member of the serine protease family, which induces the conversion of plasminogen to plasmin. Plasmin regulates cell invasion by degrading matrix proteins such as fibronectin, type IV collagen, and laminin or indirectly by activating matrix metalloproteinases (1). Previous reports indicated that uPA plays a significant role in tumor growth and metastasis (1, 2). The signaling pathways by which hypoxia/ reoxygenation controls uPA secretion through activation of NFκB in breast cancer cells are not well defined.

In this study, we demonstrate the involvement of p56<sup>lck</sup> in the redox-mediated nuclear translocation of NFκB, NFκB-DNA binding, transactivation of NFκB, and NFκB-dependent uPA promoter activity following H/R in MCF-7 and MDA-MB-231 cells. We also showed that H/R induces phosphorylation of p56<sup>lck</sup> and stimulates Lck-mediated NFκB activation through tyrosine phosphorylation of IκBα. These lead to the induction of uPA secretion, and all of these ultimately control the cell motility, invasiveness, and metastatic spread of breast cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—The rabbit polyclonal anti-p56<sup>lck</sup>, anti-Fyn, anti-NFκB p65, anti-IκBα, anti-actin and mouse monoclonal anti-phosphotyrosine, anti-phospho-IκBα antibodies, and recombinant IκBα protein were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-Lyn antibody was from BD Biosciences. The mouse monoclonal anti-uPA antibody was obtained from Oncogene. 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (pp6), 6-(methyl-1,3,5-trihydroxanthraquinone (emodin), SH2 domain inhibitor peptide (Ac-Asp-Tyr(P0,H<sub>2</sub>)-Phe-Pro-Leu-Val-NH<sub>2</sub>, SN-50, and SN-50 Mkt were purchased from Calbiochem. The [γ-<sup>32</sup>P]ATP was purchased from Board of Radiation and Isotope Technology (Hyderabad, India). The dual luciferase reporter assay system was obtained from Promega. LipofectAMINE Plus reagent was purchased from Invitrogen. The FITC-conjugated goat anti-rabbit IgG was from Pharmingen. Boyden type cell migration chambers were obtained from Corning. All other chemicals were analytical grade.

**Cell Culture**—The MDA-MB-231 and MCF-7 cells were purchased from ATCC (Manassas, VA). Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium. The media were supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C.

**Hypoxic Cultures**—The MCF-7 and MDA-MB-231 cells grown to 50-70% confluence were made hypoxic in evacuation chambers by intermittent application of vacuum and sparging with 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Cells were analyzed at this point or maintained under hypoxic conditions in presence of 100 mM dithionite (an oxygen scavenger) at 37 °C for 0-24 h. Under these conditions, oxygen levels drop to <10 mm Hg. Cells were either harvested or reoxygenated for the indicated periods of time by replacing the medium with fresh medium and incubating the cultures in humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C.

**Plasmids and DNA Transfection**—Wild type Lck and mutant Lck F394 (mutated at tyrosine 394 to phenylalanine) cDNAs in an expression vector (pCEP4) were a generous gift from Dr. Bartholomew M. Sefton (Salk Institute, La Jolla, CA). The luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB binding site was a kind gift from Dr. Jean de Martigny (University of Vienna, Vienna, Austria). Full-length human uPA promoter (−2062 to +27) in luciferase reporter gene plasmid pGL2 basic was a generous gift from Dr. Ute Reuning (Universitaet Muenchen, Germany). Both MCF-7 and MDA-MB-231 cells were transiently transfected with each of the above cDNA construct using LipofectAMINE Plus according to the instructions from the manufacturer (Invitrogen). Briefly, cDNA (8 μg) was mixed with Plus reagent, and then cDNA Plus reagent was incubated with LipofectAMINE. The LipofectAMINE Plus cDNA complex was added to the cells and incubated further at 37 °C for 12 h. The control cells received LipofectAMINE Plus alone. The cell viability was detected by a trypan blue dye exclusion test. After incubation, medium was removed, and the cells were reseeded with fresh medium and maintained for an additional 12 h. These transfected cells were individually or in combination used for in vivo kinase assay, interaction studies between Lck and tyrosine-phosphorylated IκBα, EMSA, luciferase reporter gene assay, cell migration assay, and detection of uPA expression by Western blot analysis.

**Immunoprecipitation and in vitro Kinase Assay**—Both MCF-7 and MDA-MB-231 cells were individually induced by hypoxia for 0-24 h and reoxygenated for 90 min. Cells were lysed in lysis buffer (1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA). The protein concentrations in the cleared lysates were measured by Bio-Rad protein assay. The equal amount of total proteins in lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody, and half of the immunoprecipitated samples were resolved by SDS-PAGE and analyzed by Western blot using mouse anti-phosphotyrosine antibody. The remaining half of the samples was analyzed by Western blot using anti-Lck antibody. In separate experiments, both these cells were exposed with hypoxia for 3 h and then induced by H/R for 0-135 min. Cells were lysed and immunoprecipitated with rabbit polyclonal anti-Lck antibody. The levels of phospho- and non-phospho-Lck in the immunoprecipitated samples were detected by Western blot analysis using mouse monoclonal anti-phosphotyrosine and rabbit polyclonal anti-Lck antibodies, respectively. As loading control, the expression of actin was analyzed by Western blot using anti-actin antibody.

In other experiments, these cells were transiently transfected with wild type Lck or mutant Lck F394 in pCEP4 in presence of LipofectAMINE Plus. These cells were exposed with hypoxia for 3 h and...
then induced by H/R for 90 min. These transfected cells were immunoprecipitated with anti-Lck antibody, and tyrosine phosphorylation of Lck was detected by immunoblotting with anti-phosphotyrosine antibody. The same blots were reprobed with rabbit polyclonal Lck inhibitory peptides and then induced by H/R as described above. These cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody, and the levels of tyrosine-phosphorylated IκBα and IκBβ were detected by Western blot analysis using anti-phosphotyrosine and anti-IκBα antibodies, respectively.

To check whether tyrosine 384 of Lck is responsible for interaction with phosphorylated IκBα, both MCF-7 and MDA-MB-231 cells were individually transfected with wild type or mutant Lck F394 in presence of LipofectAMINE Plus and then induced by H/R. These cell lysates were immunoprecipitated with anti-Lck antibody and analyzed by Western blot using anti-IκBα or anti-phosphotyrosine antibody. To delineate the effect of Lck inhibitors (emodin or pp2) on these interactions, these cells were individually cotransfected with different concentrations of emodin (0–10 μM) or pp2 (0–4 nM) and then induced by H/R as described above. These cells were immunoprecipitated with anti-Lck antibody and immunoblotted with anti-IκBα antibody. As loading control, the expression of actin was analyzed by Western blot using anti-actin antibody.

**Immunofluorescence Study**—Both MCF-7 and MDA-MB-231 cells were grown in monolayer on silicon-coated glass slides and then induced by hypoxia for 3 h and reoxygenated for 0–120 min. In separate experiments, cells were pretreated with various concentrations of emodin (0–10 μM) or pp2 (0–4 nM) at 37 °C for 6 h and then induced by hypoxia for 3 h followed by reoxygenation for 90 min. The cells were fixed for 15 min, blocked with 3% BSA in PBS for 30 min, and washed with phosphate-buffered saline (pH 7.4). The fixed cells were incubated with rabbit polyclonal anti-NFκB, p65 antibody (1:100 dilution) at room temperature for 2 h. The cells were washed with phosphate-buffered saline (pH 7.4) and incubated with FITC-conjugated anti rabbit IgG for 1 h at room temperature. The cells were washed, mounted with coverslips, and analyzed under confocal microscopy (Zeiss).

**NFκB Luciferase Reporter Gene Assay**—The semi-confluent MCF-7 and MDA-MB-231 cells grown in 6-well plates were individually transfected with a luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB-binding site (a generous gift from Dr. Robert Herbst, Vetter, Vienna) using LipofectAMINE Plus reagent. The transfection efficiency was normalized by cotransferring the cells with pRL vector (Promega) containing a full-length Renilla luciferase gene under the control of a constitutive promoter. These transfected cells were induced by hypoxia for 3 h and reoxygenated for 24 h. In separate experiments, both these cells transfected with pNFκB-Luc were treated with varying concentrations of emodin (0–16 μM) or pp2 (0–4 nM) at 37 °C for 6 h and then induced by H/R as described above. In another experiments, these cells were transiently cotransfected with pNFκB-Luc and wild type Lck or Lck F394 in pCEP4 and then induced by H/R. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by luminescence (Promega) using the dual luciferase assay system according to the instructions from the manufacturer (Promega). Changes in luciferase activity with respect to control were calculated.

**EMSA**—Electrophoretic mobility shift assay was performed as described previously (41). Briefly, MCF-7 and MDA-MB-231 cells were induced by hypoxia for 3 h followed by reoxygenation for 90 min. In separate experiments, cells were transiently transfected with wild type Lck or Lck F394 in presence of LipofectAMINE Plus or pretreated with Lck inhibitors (emodin and pp2) and then induced by H/R as described above. The nuclear extracts were prepared, and the extracts (10 μg) were incubated with 16 fmol of 5′-labeled double-stranded NFκB b oli-gonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG-3′) in binding buffer for 15 min at room temperature (Upstate). Nondenat P-40, 5% glycerol, and 50 mM NaCl containing 2 μg of salmon sperm DNA. The DNA–protein complex was resolved by a native polyacrylamide gel and analyzed by autoradiography. For super shift assay, the nuclear extracts from MDA-MB-231 cells were incubated with anti-p65 antibody for 30 min at room temperature and analyzed by EMSA. **Urea Promoter Construct and Luciferase Reporter Gene Assay**—The full-length human uPA-promoter (−2602 to +27) was cloned into the luciferase reporter gene plasmid, pGL2-Basic. Both MCF-7 and MDA-MB-231 cells were individually cotransfected with uPA promoter-driven luciferase reporter plasmid pGL2-basic and pRL vector containing full-length Renilla luciferase gene under the control of constitutive promoter vectors. The transfected cells were transfected with pGL2-basic and pRL vector containing full-length Renilla luciferase gene under the control of constitutive promoter for 24 h and then tested for luciferase activity. The luciferase activity (Renilla luciferase activity/cytosine nucleotide (5′-AGT TGA GGG GAC TTT CCC AGG-3′) in binding buffer for 15 min at room temperature (Upstate). Nondenat P-40, 5% glycerol, and 50 mM NaCl containing 2 μg of salmon sperm DNA. The DNA–protein complex was resolved by a native polyacrylamide gel and analyzed by autoradiography. For super shift assay, the nuclear extracts from MDA-MB-231 cells were incubated with anti-p65 antibody for 30 min at room temperature and analyzed by EMSA. **Urea Promoter Construct and Luciferase Reporter Gene Assay**—The full-length human uPA-promoter (−2602 to +27) was cloned into the luciferase reporter gene plasmid, pGL2-Basic. Both MCF-7 and MDA-MB-231 cells were individually cotransfected with uPA promoter-driven luciferase reporter plasmid pGL2-basic and pRL vector containing full-length Renilla luciferase gene under the control of constitutive promoter vectors. The transfected cells were transfected with pGL2-basic and pRL vector containing full-length Renilla luciferase gene under the control of constitutive promoter for 24 h and then tested for luciferase activity. The luciferase activity (Renilla luciferase activity/cytosine nucleotide (5′-AGT TGA GGG GAC TTT CCC AGG-3′) in binding buffer for 15 min at room temperature (Upstate). Nondenat P-40, 5% glycerol, and 50 mM NaCl containing 2 μg of salmon sperm DNA. The DNA–protein complex was resolved by a native polyacrylamide gel and analyzed by autoradiography. For super shift assay, the nuclear extracts from MDA-MB-231 cells were incubated with anti-p65 antibody for 30 min at room temperature and analyzed by EMSA.
**RESULTS**

**H/R Induces Tyrosine Phosphorylation of Lck and Lck Kinase Activity in Breast Cancer Cells**—We have investigated the effects of H/R on Lck-dependent NFκB-mediated uPA secretion through tyrosine phosphorylation of Lck in breast cancer cells. Accordingly, we first checked whether H/R is able to induce tyrosine phosphorylation of Lck in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. Both MCF-7 and MDA-MB-231 cells were individually induced by hypoxia for 0–24 h and reoxygenated for 90 min. Cells were lysed, and equal amounts of total proteins from the cell lysates were subjected to immunoprecipitation with rabbit polyclonal anti-Lck antibody. The immunocomplex was resolved by SDS-PAGE and detected by Western blot analysis using anti-uPA antibody. Our data revealed that maximum tyrosine phosphorylation of Lck is observed when cells are reoxygenated for 75 min and sustained up to 105 min.

**H/R Regulates p56\(^{lck}\) and NFκB-dependent uPA Secretion**
phosphorylation of Lck in a time-dependent manner, both these cells were induced by hypoxia for 3 h and reoxygenated for 0–135 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody, and immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody and anti-Lck antibodies, respectively. The results showed that maximum tyrosine phosphorylation of Lck was observed at 75 min in cell lysates immunoprecipitated with anti-Lck antibody. Half of the immunoprecipitated samples were incubated with anti-Lck antibodies, and the remaining half were incubated with 2 μCi of [γ-32P]ATP and recombinant IgG as substrate in kinase assay buffer as described under “Experimental Procedures.” In separate experiments, cell lysates immunoprecipitated with anti-Lck antibody were incubated with TP and used for kinase assay. Note the cell lysates immunoprecipitated with anti-Lck antibody showed the kinase activity using IgG as substrate in presence of H/R, and it is inhibited by Lck inhibitors (upper panels of C and D). Half of the immunoprecipitated samples (using anti-Lck, anti-Lyn, or anti-Fyn antibody) containing equal amount of total proteins were analyzed by Western blot using anti-Lck antibody, and the immunoprecipitates were incubated with 2 μCi of [γ-32P]ATP and recombinant IgG as substrate in kinase assay buffer as described under “Experimental Procedures.” The results showed that maximum tyrosine phosphorylation of Lck was observed at 75 min and sustained up to 105 min in both MCF-7 (Fig. 1C) and MDA-MB-231 (Fig. 1D) cells (upper panels, lanes 1–9). The expression of non-phospho-Lck remained unchanged (middle panel, lanes 1–9). Actin was used as loading controls (Fig. 1A–D, lower panels). The bands were quantified by densitometric analysis and normalized with respect to actin. The values of fold changes are indicated.

To further confirm whether tyrosine 394 of Lck is involved in H/R-induced tyrosine phosphorylation; these cells were transfected with wild type Lck and mutant Lck F394, induced by hypoxia for 3 h, and reoxygenated for 90 min. These cells were lysed and immunoprecipulated with anti-Lck antibody. Half of the immunoprecipitated samples were immunoblotted with anti-phosphotyrosine antibody, and the remaining half of the samples were immunoblotted with anti-Lck antibody. The data revealed that MCF-7 (Fig. 2A) and MDA-MB-231 (Fig. 2B) cells transfected with wild type Lck enhances the tyrosine phosphorylation of Lck (upper panels, lane 3) compared with cells transfected with LipofectAMINE Plus alone (lane 2) or mutant Lck F394 transfected cells (lane 4). As expected, no tyrosine-phosphorylated Lck-specific band was detected in absence of H/R. The level of expression of non-phosphorylated Lck was almost identical in non-transfected and transfected cells (upper panels, lanes 1–4). The bands were quantified by densitometric analysis and normalized with actin. The values of fold changes are indicated. There were at least 3-fold increases in tyrosine phosphorylation of Lck in wild type Lck-transfected cells compared with cells transfected with mutant Lck F394 or cells induced with H/R alone, suggesting that tyrosine 394 of Lck is crucial for phosphorylation.

To assess the kinase activity of Lck, Lyn and Fyn upon H/R induction, both MCF-7 and MDA-MB-231 cells were induced by hypoxia for 3 h and reoxygenated for 90 min; the lysates were individually immunoprecipitated with anti-Lck, anti-Lyn, or anti-Fyn antibodies; and kinase assay was performed. The immunoprecipitated samples were incubated with [γ-32P]ATP and recombinant IgG in kinase assay buffer. The samples were resolved by SDS-PAGE and autoradiographed. The data showed that Lck phosphorylates IgG in both MCF-7 (Fig. 2C, upper panel, lane 2) and MDA-MB-231 (Fig. 2D, upper panel, lane 2) cells, whereas this specific phosphorylation was absent in cell lysates immunoprecipitated with anti-Lyn (lane 6) or...
anti-Fyn (lane 7) antibody. These results demonstrated that Lck plays significant role in H/R-induced tyrosine phosphorylation of IkBα in these cells. To delineate the specificity of Lck on phosphorylation of IkBα, both these cells were pretreated with emodin on phosphorylation and degradation of IkBα, these cells were induced by H/R, and then kinase assay was performed. The data revealed that both emodin and pp2 blocked the H/R-induced Lck kinase activity using IkBα as substrate in MCF-7 and MDA-MB-231 cells (lanes 4 and 5). To further confirm the specificity of tyrosine phosphorylation of IkBα by Lck, both these cell lysates were immunoprecipitated with anti-Lck antibody and immunoprecipitated samples were used for kinase assay in presence of TP, which specifically inhibits the tyrosine phosphorylation. The data indicated that TP inhibited the H/R-induced Lck-dependent tyrosine phosphorylation of IkBα in these cells (lane 8). As expected, no phosphorylation of IkBα was observed in cells grown under normoxic condition (lane 1) or cells induced by H/R but without immunoprecipitation with anti-Lck antibody (lane 3). Half of the immunoprecipitated samples (using anti-Lck, anti-Lyn, or anti-Fyn antibody) containing equal amount of total proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Lck, anti-Lyn, or anti-Fyn antibody to ensure the equal amount of loading. These results showed that Lck, Lyn, and Fyn are expressed in these cells and an equal amount of total protein was used for kinase assay (Fig. 2, C and D, lower panel, lanes 1–8). These data further demonstrated that H/R induces the tyrosine phosphorylation of IkBα by inducing the Lck but not Lyn or Fyn kinase activity in these cells.

H/R Stimulates Tyrosine but Not Serine Phosphorylation of IkBα—To delineate the role of H/R on tyrosine phosphorylation of IkBα, both MCF-7 and MDA-MB-231 cells were exposed with hypoxia for 0 to 24 h and reoxygenated for 90 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-IkBα antibody, and the immunoprecipitated samples were analyzed by Western blot using mouse monoclonal anti-phosphotyrosine antibody. The tyrosine phosphorylation of IkBα was detected after 1 h of exposure with hypoxia, whereas maximum tyrosine phosphorylation was found at 3 h of exposure with hypoxia followed by reoxygenation in both MCF-7 (Fig. 3A, panel a, lanes 1–7) and MDA-MB-231 (Fig. 3B, panel a, lanes 1–7) cells. Previous reports indicated that various cytokines and other factors play major roles in serine phosphorylation and degradation of IkBα; therefore, we sought to determine whether H/R has any effect on serine phosphorylation and degradation of IkBα in breast cancer cells. Accordingly, cells were induced by H/R and cell lysates were immunoprecipitated with anti-IkBα antibody. The immunoprecipitated samples were immunoblotted with mouse monoclonal anti-phosphoserine IkBα antibody. Same blots were reprobed with rabbit polyclonal anti-IkBα antibody. H/R had no effect on serine phosphorylation and degradation of IkBα in MCF-7 (Fig. 3A, panels b and c, lanes 1–7) and MDA-MB-231 (Fig. 3B, panels b and c, lanes 1–7) cells. Actin was used as loading controls (panel d, lanes 1–7). The bands were quantified by densitometric analysis and normalized with respect to actin. The values of -fold changes are indicated.

To examine the effect of reoxygenation on IkBα tyrosine phosphorylation in a time-dependent manner, both MCF-7 and MDA-MB-231 cells were induced by hypoxia for 3 h and reoxygenated for 0–135 min. Cell lysates containing equal amount of total proteins were subjected to immunoprecipitation with rabbit polyclonal anti-IkBα antibody. The immunocomplex was resolved by SDS-PAGE and detected by Western blot analysis using mouse monoclonal anti-phosphotyrosine antibody (Fig. 3, C and D, panels a, lanes 1–9). The data revealed that maximum tyrosine phosphorylation of IkBα started at 75 min and was sustained up to 90 min upon reoxygenation in case of MCF-7 cells (Fig. 3C, panel a, lanes 5 and 6) and initiated at 60 min and remained up to 135 min in MDA-MB-231 cells (Fig. 3D, panel a, lanes 4–9). To check whether H/R had any effect on serine phosphorylation and degradation of IkBα, both these cells were induced by H/R and cell lysates were immunoprecipitated with anti-IkBα antibody. The immunoprecipitated samples were separated by SDS-PAGE and immunoblotted individually with mouse monoclonal anti-phosphoserine IkBα and anti-IkBα antibodies, respectively. These data suggested that H/R had no effect on serine phosphorylation and degradation of IkBα in both these cells (Fig. 3, C and D, panels b and c, lanes 1–9). Actin was used as loading controls (Fig. 3, C and D, panel d, lanes 1–9). To further confirm whether H/R regulates tyrosine phosphorylation but not serine phosphorylation and degradation of IkBα, both these cells were treated with PMA (10 ng/ml) or TNFα (0.1 nM), which are known to induce serine phosphorylation and degradation of IkBα. Cell lysates containing equal amount of total proteins were individually analyzed by Western blot using anti-phosphoserine IkBα antibody. The same blots were reprobed with rabbit polyclonal anti-IkBα antibody. The data showed that PMA (Fig. 3E) and TNFα (Fig. 3F) independently induce serine phosphorylation and degradation of IkBα in MDA-MB-231 (panels a and b, lanes 1–9) cells. Maximum PMA or TNFα induced serine phosphorylation of IkBα was observed at 15 min, whereas maximum IkBα degradation was noted at 30 min in these cells. Similar results were obtained in MCF-7 cells (data not shown). Actin was used as loading controls (panel c, lanes 1–9). All of these bands were quantified densitometrically, and the values of -fold changes are calculated. These results clearly suggested that H/R induces tyrosine phosphorylation but not serine phosphorylation and degradation of IkBα in these cells.

H/R Enhances Lck-dependent Tyrosine Phosphorylation of IkBα—To delineate whether Lck is involved in tyrosine phosphorylation of IkBα in presence of H/R, both MCF-7 and MDA-MB-231 cells were transiently transfected with wild type or mutant Lck F394 and induced by hypoxia for 3 h followed by reoxygenation for 90 min. Cell lysates were immunoprecipitated with anti-IkBα antibody. The immunoprecipitated samples were detected by Western blot analysis using anti-phosphotyrosine antibody. The data showed that cells transfected with wild type Lck induced the tyrosine phosphorylation of IkBα at least 3-fold in MCF-7 cells (Fig. 4A, panel a, lanes 3) and at least 2-fold in MDA-MB-231 cells (Fig. 4B, panel a, lanes 3) compared with cells transfected with LipofectAMINE Plus alone (lane 2) or mutant Lck F394-transfected cells (lane 4) in presence of H/R. As expected, in the absence of H/R, no tyrosine phosphorylation of IkBα was observed (lane 1). The serine phosphorylation and degradation of IkBα were also detected by Western blot using anti-phosphoserine-IkBα and anti-IkBα antibodies, respectively. The results indicated that transfection of these cells with wild type or mutated Lck followed by treatment with H/R have no effect on serine phosphorylation and degradation of IkBα (Fig. 4, A and B, panels b and c, lanes 1–4). Actin-specific band was shown as loading controls (panel d, lanes 1–4).

To check the effect of Lck inhibitors (emodin and pp2) on H/R-induced tyrosine phosphorylation of IkBα, these cells were individually pretreated with different concentrations of emodin or pp2 and then induced by H/R as described above. Cell lysates were immunoprecipitated with anti-IkBα antibody and detected by Western blot analysis using mouse monoclonal anti-phosphotyrosine antibody. The results revealed that H/R-induced tyrosine phosphorylation of IkBα (Fig. 4, C and D, panel a, lane 2) was inhibited when increasing concentrations of...
emodin (panel a, lanes 3 and 4) or pp2 (panel a, lanes 5 and 6) were used. As expected, no tyrosine-phosphorylated IκBα specific band was detected in non-H/R-induced cells (lane 1). Both emodin and pp2 had no effects on serine phosphorylation (Fig. 4, C and D, panel b, lanes 1–6) and degradation (panel c, lanes 1–6) of IκBα in presence of H/R in these cells. Actin was used as loading control (panel d, lanes 1–6). These data clearly demonstrated that Lck plays significant role in H/R-induced tyrosine phosphorylation but not serine phosphorylation of IκBα in these cells. All of these bands were quantified by densitometric analysis, and the values of -fold changes are indicated.

**H/R Induces the Interaction between SH2 Domain of Lck and Tyrosine-phosphorylated IκBα**—To delineate the role of H/R or other agents in regulation of direct interaction between SH2 domain of Lck and tyrosine-phosphorylated IκBα, both these cells were individually treated with pV (250 μM) for 20 min, H2O2 (5 μM) for 20 min, or induced by hypoxia for 3 h and reoxygenated for 90 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-IκBα antibody, separated by SDS-PAGE, and an-

**Fig. 3. H/R induces IκBα tyrosine phosphorylation.** A and B, MCF-7 (A) and MDA-MB-231 (B) cells were exposed with hypoxia for 0–24 h and reoxygenated for 90 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-IκBα antibody, and half of the immunoprecipitated samples were analyzed by Western blot using mouse monoclonal anti-phosphotyrosine antibody. The remaining half of the immunoprecipitated samples were immunoblotted with mouse monoclonal anti-phospho-IκBα antibody. Same blots were reprobed with rabbit polyclonal anti-IκBα antibody. Maximum tyrosine phosphorylation of IκBα was detected at 3 h (panels a of A and B, lanes 1–7). Serine phosphorylation of IκBα and non-phospho IκBα were remained unchanged (panels b and c of A and B, lanes 1–7). Actin was used as loading control (panels d of A and B). C and D, both MCF-7 (C) and MDA-MB-231 (D) cells were exposed with hypoxia for 3 h and reoxygenated for 0–135 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-IκBα antibody. The immunocomplex was individually analyzed by Western blot using mouse monoclonal anti-phosphotyrosine (panels a of C and D, lanes 1–9), mouse monoclonal anti-phosphoserine IκBα (panels b of C and D, lanes 1–9), and rabbit polyclonal anti-IκBα (panels c of C and D, lanes 1–9) antibodies. Note that H/R induces maximum tyrosine phosphorylation at 75–90 min, but there were no changes in serine phosphorylation and degradation of IκBα in these cells. Actin was used as loading control (panels d of C and D). E and F, PMA (E) and TNFα (F) induce serine phosphorylation and degradation of IκBα. MDA-MB-231 cells were treated with PMA (10 ng/ml) or TNFα (0.1 nm) for 0–135 min. Cell lysates containing equal amount of total proteins were individually analyzed by Western blot using mouse monoclonal anti-phospho-IκBα antibody (panels a of E and F, lanes 1–9). The same blots were reprobed with anti-IκBα (panels b of E and F, lanes 1–9) and anti-actin (panels c of E and F, lanes 1–9) antibodies, respectively. Note that both PMA and TNFα induce serine phosphorylation of IκBα at 15 min (panels a of E and F, lane 2), whereas maximum degradation was occurred at 30 min (panels b of E and F, lane 3) in these cells. All these bands were quantified densitometrically, and the values of -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.
Western blot using anti-1xBa or anti-phosphotyrosine antibody. The data showed that H/R-induced interaction between tyrosine-phosphorylated 1xBa and Lck (Fig. 5, C and D, upper and middle panels, lane 2) was drastically reduced by SH2 domain inhibitory peptide in a dose-dependent manner (upper and middle panels, lanes 3 and 4), suggesting that SH2 domain of Lck is involved in this interaction. No 1xBa-specific bands were detected in absence of H/R (upper and middle panels, lane 1).

To delineate the effect of genetic (Lck F394) and pharmacological (emodin or pp2) inhibitors of Lck on H/R-induced interaction between SH2 domain of Lck and tyrosine-phosphorylated 1xBa, both these cells were either transfected with wild type or mutant Lck F394 and then induced by H/R. Similarly, these cells were pretreated with different concentrations of emodin (0–10 μM) or pp2 (0–4 μM) and induced by H/R. Cell lysates were immunoprecipitated with anti-Lck antibody and detected by Western blot using anti-1xBa or anti-phosphotyrosine antibody. The data suggested that wild type Lck but not Lck F394 induces the interaction between Lck and tyrosine-phosphorylated 1xBa (Fig. 5, E and F, upper and middle panels, lane 3) compared with cells transfected with LipofectAMINE Plus alone (lane 2) or Lck F394-transfected cells (lane 4). No band was detected in non-H/R-induced cells (lane 1). Similarly, both emodin and pp2 dose-dependently suppressed the H/R-induced interaction between Lck and 1xBa (Fig. 5, G and H, upper panels, lanes 2–6). No band was observed in the absence of H/R (lane 1). Actin was used as loading controls (Fig. 5, A–H, lower panels). All these bands were quantified by densitometric analysis, and the values of -fold changes are indicated.

**H/R Stimulates Nuclear Translocation and Transactivation of NFκB through Lck-mediated Pathway**—To examine the effect of H/R on NFκB nuclear translocation, both these cells were grown on glass slides, induced by hypoxia for 3 h, and reoxygenated for 0–120 min. Cells were fixed and incubated with rabbit polyclonal anti-NFκB p65 antibody. These cells were further incubated with FITC-conjugated anti-rabbit IgG. Similarly, to check the effect of Lck inhibitors (emodin or pp2) on H/R-induced NFκB nuclear translocation, both these cells were treated with these inhibitors and then induced by H/R. These cells were incubated with anti-NFκB p65 antibody and then with FITC-conjugated anti-rabbit IgG. The data showed that H/R-induced nuclear translocation of p65 started at 60 min and was sustained up to 90 min, whereas it shuttled back into the cytoplasm at 120 min in MCF-7 (Fig. 6, panel A, a–d) and in MDA-MB-231 (panel B, a–d) cells. Both emodin and pp2 dose-dependently suppressed the H/R-induced tyrosine phosphorylation of 1xBa in these cells (panels a in C and D, lanes 1–6). Actin was used as loading control (panels d in C and D). All of these bands were quantified by densitometric analysis, and the values of -fold changes are indicated. The results shown here represent three experiments exhibiting similar effects.
In other experiments, cells transfected with pNFκB-Luc were treated with different doses of Lck inhibitors (emodin or pp2). All these cells were induced by hypoxia for 3 h and reoxygenated for 24 h. Cells were harvested in passive lysis buffer and used to measure the luciferase activity. The data demonstrated that wild type Lck enhanced the NFκB transcriptional activity compared with cells transfected with LipofectAMINE Plus alone or Lck F394-transfected cells in presence of H/R ([Fig. 6, C and D]). Both emodin and pp2 inhibited the H/R-induced NFκB transactivation in a dose-dependent manner ([Fig. 6, C and D]). The values were normalized to Renilla luciferase activity. The -fold changes were calculated, and the results are expressed as the means ± S.E. of three determination. The values were also analyzed by Student’s t test ($p < 0.001$).

**H/R Induces Lck-dependent NFκB-DNA Binding and uPA Promoter Activity**—In previous figures ([Fig. 6]), we have demonstrated that H/R induces Lck-dependent NFκB nuclear translocation and transactivation. Therefore we sought to determine the role of Lck on H/R-induced NFκB-DNA binding. Accordingly, both MCF-7 ([panel A]) and MDA-MB-231 ([panel B]) cells were transiently transfected with wild type Lck or mutant Lck F394 plasmid or pretreated with Lck inhibitors (emodin or pp2) and then induced with hypoxia for 3 h and reoxygenation for 90 min. The nuclear extracts were prepared and analyzed by EMSA. The results demonstrated that H/R induces NFκB-DNA binding ([Fig. 7, panels A and B, lane 2]) compared with cells grown under normoxic condition ([lane 1]). Wild type Lck-transfected cells showed much higher level of NFκB-DNA binding ([lane 4]) compared with Lck F394-transfected cells ([lane 3]). Similarly, both emodin and pp2 inhibited H/R-induced NFκB-DNA binding in both these cell types ([lanes 5 and 6]). These results clearly demonstrated that H/R induces Lck-dependent NFκB-DNA binding in these cells. To further confirm that the band obtained ([panels A and B]) by EMSA in H/R-induced cells
is indeed NFκB, the nuclear extracts from MDA-MB-231 cells were incubated with anti-p65 antibody, incubated further with FITC-conjugated anti-rabbit IgG, and analyzed under confocal microscopy. Panel a, untreated cells; panel b, reoxygenation for 60 min; panel c, reoxygenation for 90 min; panel d, reoxygenation for 120 min; panel e, treated with emodin (5 μM) and then with H/R; panel f, treated with emodin (10 μM) and then with H/R; panel g, treated with pp2 (2 nM) and then with H/R; panel h, treated with pp2 (4 nM) and then with H/R. Note that there was H/R-induced translocation of p65 at 90 min in both MCF-7 and MDA-MB-231 cells, but it was shuttled back at 120 min. Both emodin and pp2 inhibited the H/R-induced nuclear translocation of p65. C and D, H/R stimulates Lck-mediated NFκB transactivation. Both MCF-7 (C) and MDA-MB-231 (D) cells were transiently transfected with luciferase reporter construct (pNFκB-Luc) in the presence of LipofectAMINE Plus as described under “Experimental Procedures.” In separate experiments, these transfected cells were treated with Lck inhibitors (emodin or pp2). In another experiment, these cells were transfected with wild type Lck or mutant Lck F394 along with pNFκB-Luc. The transfection efficiency was normalized by cotransferring the cells with pRL vector, induced by hypoxia for 3 h, and reoxygenated for 24 h, and luciferase activity was measured. The cells transfected with wild type Lck followed by induction with H/R showed maximum pNFκB luciferase activity compared with mutant Lck F394-transfected cells or cells transfected with LipofectAMINE Plus alone. Both emodin and pp2 dose-dependently suppressed the H/R-induced pNFκB luciferase activity (panels C and D). The values were normalized to Renilla luciferase activity. The -fold changes were calculated, and the results are expressed as the means ± S.E. of three determinations. The values were also analyzed by Student’s t test (*, p < 0.001).
tides (SN-50 or SN-50M). The cells were induced with H/R for 24 h, and luciferase activity was measured. The results shown that NFκB inhibitory peptide SN-50 inhibited H/R-induced uPA promoter activity in both these cells. These data suggested that NFκB-responsive elements but not regulatory elements are essential for H/R-induced Lck-dependent uPA promoter activity in these cells.

**H/R Stimulates Lck-dependent NFκB-mediated uPA Secretion**—Because it is documented that NFκB-responsive element is present in the promoter region of uPA and we have shown in Fig. 7 that H/R stimulates NFκB-dependent uPA promoter activity, we sought to determine the impact of H/R on NFκB-mediated uPA secretion in MCF-7 and MDA-MB-231 cells. Accordingly, these cells were subjected to hypoxia for 3 h and reoxygenation for 0–16 h. Cell lysates containing equal amount of total proteins were resolved by SDS-PAGE and detected by Western blot analysis using mouse monoclonal anti-uPA antibody. The data indicated that there were at least 7.2- and 7.8-fold increases in uPA secretion in MCF-7 (Fig. 8A, upper panel, lanes 1–9) and MDA-MB-231 (Fig. 8B, upper panel, lanes 1–9) cells, respectively, when induced by H/R for 16 h compared with control. However, the rate of induction of overall uPA secretion was significantly higher in MDA-MB-231 cells compared with MCF-7 cells.

To examine the role of Lck on H/R-induced uPA expression, these cells were individually pretreated with different concentrations of emodin (0–16 μM) or pp2 (0–4 nM). Both these cells were then induced by H/R, and the level of uPA in the cell lysates was detected by Western blot analysis using anti-uPA antibody. The data indicated that both emodin and pp2 dose-dependently inhibited H/R-induced uPA secretion in MCF-7 (Fig. 8C, upper panels, lanes 1–8) and MDA-MB-231 (Fig. 8D, upper panels, lanes 1–8) cells. In separate experiments, these cells were transfected with wild type Lck or mutant Lck F394.
in pCEP4 and then induced by H/R. The data revealed that cells transfected with wild type Lck showed maximum uPA secretion compared with LipofectAMINE Plus-transfected cells or cells transfected with mutant Lck F394 (Fig. 8, E (MCF-7) and F (MDA-MB-231), upper panels, lanes 1–4).

We have also investigated the effect of NF-κB inhibitory peptide (SN-50) on H/R-induced uPA expression in these cells. Both these cells were either treated with SN-50 or SN-50M, induced by H/R, and uPA level was detected by Western blot analysis. The data indicated that SN-50 but not control peptide SN-50M suppressed the H/R-induced uPA secretion in MCF-7 (Fig. 8G, upper panel, lanes 1–4) and MDA-MB-231 (Fig. 8H, upper panel, lanes 1–4) cells. All these blots were reprobed with anti-actin antibody as loading controls (Fig. 8, A–H, lower panels). All these bands were quantified by densitometric analysis, and the values of -fold changes are indicated.

H/R Enhances Lck and NFκB-dependent uPA-mediated Cell Migration—Because H/R regulates Lck-dependent NFκB activation and uPA secretion in MCF-7 and MDA-MB-231 cells, we sought to determine whether H/R-regulated Lck/NFκB-dependent uPA expression has any role in breast cancer cell migration. Accordingly, both MCF-7 and MDA-MB-231 cells were individually pretreated with emodin, pp2, SN-50, SN-50M, and uPA antibody. In separate experiments, these cells were separately transfected with wild type Lck or mutant Lck F394 in presence of LipofectAMINE Plus. These cells were induced by hypoxia for 3 h, reoxygenated for 16 h, and used for migration assay. H/R induces the cell migration in both MCF-7 (565%) and MDA-MB-231 (710%) cells compared with cells grown under normoxic condition (100%). Wild type Lck-transfected cells showed maximum migration in MCF-7 (880%) and in MDA-MB-231 (1076%) cells compared with MCF-7 (558%) or MDA-MB-231 (697%) cells transfected with mutant Lck F394. Emodin suppressed H/R-induced cell migration in a dose-de-
FIG. 9. **A** and **B**, H/R enhances Lck-mediated breast cancer cell migration. Both MCF-7 (A) and MDA-MB-231 (B) cells were either treated with Lck inhibitors (emodin and pp2) or transfected with wild type Lck or mutant Lck F394 and then induce by hypoxia for 3 h and reoxygenated for 16 h. These treated or transfected cells were used for cell migration assay as described under “Experimental Procedures.” The number of non-induced cells migrated was considered as 100%. The cell transfected with wild type Lck followed by induction with H/R showed maximum cell migration compared with Lck F394-transfected cells or cells induced by H/R alone. Both emodin and pp2 inhibited the H/R-induced cell migration in a dose-dependent manner. **C** and **D**, effects of NFκB modulators and uPA on H/R-induced cell migration. MCF-7 (C) and MDA-MB-231 (D) cells were individually treated with SN-50, SN-50M, and anti-uPA antibody and then induced by H/R as described previously. These cells were used for cell migration assay. The H/R-induced cell migration is drastically reduced by uPA antibody and SN-50 peptide, but not with SN-50M peptide. The results are expressed as the means ± S.E. of three determinations. The values were analyzed statistically by Student’s t test (*, p < 0.002).

DISCUSSION

In this report, we present evidence for the involvement of protein-tyrosine kinase, p56\textsuperscript{lck}, in the redox-regulated activation of NFκB through tyrosine phosphorylation of IκB\textalpha, uPA secretion and cell motility following H/R in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. We demonstrated that H/R induces tyrosine phosphorylation of p56\textsuperscript{lck} and nuclear translocation of NFκB, NFκB-DNA binding, and transactivation of NFκB through tyrosine phosphorylation of IκB\textalpha in these cells. Transient transfection of these cells with wild type Lck but not with mutant Lck F394 followed by H/R induces the tyrosine phosphorylation of IκB\textalpha and transcriptional activation of NFκB, and these processes are inhibited by Lck inhibitors (emodin and pp2). In vitro kinase assay indicated that immunoprecipitated p56\textsuperscript{lck} substrates but not Lyn or Fyn directly phosphorylates IκB\textalpha in presence of H/R, and this activity is blocked by Lck inhibitors. Pervanadate, H\textsubscript{2}O\textsubscript{2}, and H/R induce the interaction between Lck and tyrosine-phosphorylated IκB\textalpha, and this interaction is inhibited by SH2 domain inhibitory peptide indicating that SH2 domain of Lck is involved in this interaction. H/R stimulates NFκB-dependent uPA promoter activity and cell motility and subsequently induces uPA expression in these cells. These data demonstrated that H/R induces cell motility and tyrosine kinase, p56\textsuperscript{lck}-dependent NFκB activation, and uPA expression through tyrosine phosphorylation of IκB\textalpha in MCF-7 and MDA-MB-231 cells.

Previous results suggested that Lck acts as a proto-oncogene. The overexpression of wild type p56\textsuperscript{lck} reproducibly developed thymic tumors, indicating that p56\textsuperscript{lck} contribute to the pathogenesis of human neoplastic diseases. Earlier data demonstrated that H\textsubscript{2}O\textsubscript{2} induced p56\textsuperscript{lck} catalytic activity by phosphorylating Tyr-394 and Tyr-505. Thus dephosphorylation of Tyr-505 is not a prerequisite for either phosphorylation of Lck at Tyr-394 or catalytic activation of kinase. These data suggested that activation of Lck by phosphorylation of Tyr-394 is dominant over any inhibition induced by phosphorylation of Tyr-505 (30). However, it is unclear whether phosphorylation of Lck induced by H/R treatment is the result of activation of kinase or inhibition of a phosphatase or by both. It has been also indicated that treatment of cells with pervanadate or diamide induces Lck kinase activity and phosphorylation.
at Tyr-394 (28). It is possible that these agents may act through similar mechanisms. Our data reveals that reactive oxygen species generated by hypoxia/reoxygenation induces phosphorylation of p56\textsuperscript{Lck} at Tyr-394 and its catalytic activity.

pV induces multiple signaling pathways through Lck, Fyn, and Zap-70 in Jurkat cells (46). We have recently reported that pV induces NF\textsuperscript{κB} activation through tyrosine phosphorylation of I\textsuperscript{κB} in breast cancer cells (44). However, pV had no effect on tyrosine phosphorylation of I\textsuperscript{κB} in Lck-deficient Jurkat variants, indicating that I\textsuperscript{κB} could be phosphorylated by Lck. pV induces tyrosine phosphorylation of I\textsuperscript{κB} without degradation (37). There are evidences that tyrosine-phosphorylated I\textsuperscript{κB} at position 42 binds to SH2 domain-containing proteins like phosphatidylinositol 3'-kinase and c-Src upon treatment with pV (47). Recent report also demonstrated that H\textsubscript{2}O\textsubscript{2}, pV, or H/R induce tyrosine phosphorylation of I\textsuperscript{κBα} and NF\textsuperscript{κB} activation directly through c-Src-dependent pathways (40). However, the study also demonstrated that c-Src, Fyn, and Yes triple knock-out cells able to tyrosine-phosphorylate I\textsuperscript{κBα} upon pV treatment. This data clearly suggest that there are other tyrosine kinase(s), which are involved in this process. In our study we have demonstrated that H/R induces Lck tyrosine phosphorylation, which leads to induction of Lck kinase activity and subsequently phosphorylates tyrosine residue of I\textsuperscript{κBα} without degradation and then activates NF\textsuperscript{κB} in breast cancer cells.

The activation of transcription factor NF\textsuperscript{κB} is regulated by variety of stimuli in different cell types (31, 32). The NF\textsuperscript{κB}/Rel system has the capacity of specifically responding to various stimuli by inducing the tyrosine phosphorylation of I\textsuperscript{κBα}. Because other I\textsuperscript{κB} family proteins such as I\textsuperscript{κBβ} and I\textsuperscript{κBγ} lack tyrosine residue at position 42, involvement of these other proteins in tyrosine phosphorylation and NF\textsuperscript{κB} activation is out of the question. Our finding suggests that oxidative stress (H/R)-induced NF\textsuperscript{κB} activation does not require degradation of I\textsuperscript{κBα}. Although I\textsuperscript{κBα} is not degraded, it is most likely that it may modify during oxidative stress through another unknown mechanism, which is prerequisite for NF\textsuperscript{κB} dissociation and nuclear translocation. Although pV, a tyrosine phosphatase inhibitor, is known to activate tyrosine phosphorylation of I\textsuperscript{κBα}, the physiological significance of this phenomenon is remained unclear. H/R also induces I\textsuperscript{κBα} tyrosine phosphorylation and NF\textsuperscript{κB} nuclear translocation during tumor progression. Our studies have further demonstrated an IKK-independent pathway that regulates NF\textsuperscript{κB} through p56\textsuperscript{Lck}. Lck activated by H/R is able to phosphorylate I\textsuperscript{κBα} in vitro, and further induces NF\textsuperscript{κB} nuclear translocation and transcriptional activity. These findings clearly suggest that NF\textsuperscript{κB} activated by H/R is functionally active. In the present report, we have delineated the involvement of I\textsuperscript{κBα}/p56\textsuperscript{Lck} (protein-tyrosine kinase) pathway in mediating NF\textsuperscript{κB} transcriptional activation in breast cancer cells following hypoxia/reoxygenation.

uPA is a serine protease that plays a major role in tumor invasion, malignant progression, and distant metastasis by converting plasminogen into plasmin. There is abundant experimental evidence that uPA plays a significant role in malignant progression and tumor metastasis (1). The secretion of uPA is the prerequisite for proteolytic degradation of extracellular matrix, extracellular matrix invasion, and cell migration. An up-regulation of uPA and uPAR has been described in many human tumors. High levels of uPA and uPAR in tumor tissues are associated with poor prognosis of patients with cancer of the breast, lung, head and neck, uterine cervix, bladder, and ovary (2). It has been demonstrated that uPA is a downstream target molecule of NF\textsuperscript{κB}, because NF\textsuperscript{κB}-responsive element is present in uPA. Therefore, we sought to determine the level of cellular uPA after induction of breast cancer cells by H/R. We have demonstrated that H/R induced NF\textsuperscript{κB} activity and uPA secretion through tyrosine phosphorylation of I\textsuperscript{κBα} in these cells. The induction of uPA secretion by H/R is comparatively higher in MDA-MB-231 cells than in MCF-7 cells.

In summary, we have demonstrated for the first time that H/R induces the tyrosine phosphorylation of p56\textsuperscript{Lck} and its kinase activity. Lck is able to directly tyrosine-phosphorylate I\textsuperscript{κBα}, and this phosphorylation event is required for NF\textsuperscript{κB} activation and uPA secretion following H/R in MCF-7 and MDA-MB-231 cells. H/R also enhances the interaction between SH2 domain of Lck and I\textsuperscript{κBα} through inducing tyrosine phosphorylation of I\textsuperscript{κBα}, and this interaction is blocked by SH2 domain inhibitory peptide in these cells. Transfection of these cells with wild type Lck but not with Lck F394 stimulates the tyrosine phosphorylation of I\textsuperscript{κBα} and NF\textsuperscript{κB} transactivation in the presence of H/R, indicating that tyrosine 394 is involved in these processes. H/R induces uPA secretion and cell migration. Finally, these data demonstrated that H/R regulates p56\textsuperscript{Lck}-mediated NF\textsuperscript{κB} activation, NF\textsuperscript{κB}-dependent uPA promoter activity, and cell motility through expression of uPA in breast cancer cells (Fig. 10). These findings may be useful in designing novel therapeutic interventions that block redox-regulated p56\textsuperscript{Lck}-dependent NF\textsuperscript{κB} activation, resulting in reduction of uPA secretion and consequent blocking of cell motility, invasiveness, and metastatic spread of breast cancer.
five tandem repeats of NF-κB binding site. The full-length human uPA promoter (~2062 to ~27) in luciferase reporter gene plasmid pGL2 basic was the gift of Dr. Ute Reuning.

REFERENCES

1. Andersen, P. A., and Petersen, H. H. (2000) Cell Mol. Life Sci. 57, 25–40
2. Reuning, U., Mapolen, V., Wilhelm, O., Fischer, K., Lutz, V., Graeff, H., and Schmitt, M. (1998) Int. J. Oncol. 13, 893–906
3. Vaupel, P., Kallinowski, F., and Okunieff, P. (1989) Cancer Res. 49, 6449–6465
4. Vaupel, P., Thews, O., Kelleher, D. K., and Hockel, M. (1998) Adv. Exp. Med. Biol. 454, 591–602
5. Nordmark, M., and Overgaard, J. (2000) Radiother. Oncol. 57, 39–43
6. Nordmark, M., Lerner, J., Keller, J., Nielsen, O. S., Jennsen, O. M., Horsman, M. R., and Overgaard, J. (2001) Br. J. Cancer 84, 1070–1075
7. Hockel, M., Schienger, K., Hockel, S., and Vaupel, P. (1999) Cancer Res. 59, 4525–4528
8. Sundsfjord, K., Lyng, H., and Rosfjord, E. K. (1998) Br. J. Cancer 78, 822–827
9. Rosfjord, E. K. (2000) Int. J. Radiat. Biol. 76, 589–605
10. Dachs, G. U., and Tozer, G. M. (2000) Eur. J. Cancer 36, 1649–1660
11. Koong, A. C., Denco, N. C., Hudson, K. M., Schindler, C., Swierz, L., Koch, C., Evans, S., Ibrahim, H., Le, Q. T., Terris, D. J., and Giaccia, A. J. (2000) Cancer Res. 60, 883–887
12. Hockel, M., and Vaupel, P. (2001) J. Natl. Cancer Inst. 93, 266–276
13. Koster, A., Landgraf, S., Leipold, A., Sachse, R., Gebhart, E., Tulusan, A. H., Abraham, N., and Veillette, A. (1990) Oncogene 5, 2080–2085
14. Amundsdottir, L. T., and Leder, P. (1998) Oncogene 16, 737–746
15. Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988) Proc. Natl. Acad. Sci., U. S. A. 85, 5190–5194
16. Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) Cell 55, 301–308
17. Strauss, D. B., and Weiss, A. (1992) Cell 70, 585–599
18. Karnitz, L., Sutor, S. L., Terigoe, T., Reed, J. C., Bell, M. P., McKean, D. J., Leibson, P. J., and Abraham, R. T. (1992) Mol. Cell. Biol. 12, 4521–4530
19. Molina, T. J., Kishihara, K., Siderovski, D. P., VanEwijk, W., Narendran, A., Timms, E. Wakeham, A., Page, C. J., Hartmann, K.-U., Veillette, A., Davidson, D., and Mak, T. W. (1992) Nature 357, 161–164
20. Allen, J. M., Forbush, K. A., and Purifoy, R. M. (1992) Mol. Cell. Biol. 12, 2758–2768
21. Amrein, K. E., and Sefton, B. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4247–4251
22. Martz, J. D., Cooper, J. A., King, C. S., Ziegler, S. F., Tinker, D. A., Orelle, R. W., Krebs, E. G., and Perlmutt, R. M. (1988) Mol. Cell. Biol. 8, 540–550

23. Bergmen, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autern, M., Burn, P., and Alitalo, K. (1992) EMBO J. 11, 2919–2924
24. Casamayor, J. E., Harrison, M. L., Hellstrom, K. E., and Krebs, E. G. (1982) J. Biol. Chem. 257, 13877–13879
25. Voronova, A. F., Buss, J. E., Patschinsky, T., Hunter, T., and Sefton, B. M. (1984) Mol. Cell. Biol. 4, 2705–2713
26. Boulet, J., Fagard, R., and Fischer, S. (1987) Biochem. Biophys. Res. Commun. 149, 56–64
27. Abraham, N., and Veillette, A. (1999) Mol. Cell. Biol. 10, 5197–5206
28. Nakamura, K., Hori, T., Sato, N., sugie, K., Kawakami, T., and Yodlo, J. (1993) Oncogene 8, 3133–3139
29. Yurchak, L. K., Hardwick, J. S., Amrein, K., Perlmutter, R. M., and Sefton, B. M. (1998) J. Biol. Chem. 273, 12549–12554
30. Hardwick, J. S., and Sefton, B. M. (1995) Proc. Natl. Acad. Sci., U. S. A. 92, 4527–4531
31. Miyamoto, S., and Verma, I. M. (1995) Adv. Cancer Res. 66, 255–292
32. Ghosh, S., and Karin, M. (2002) Cell 108, 851–896
33. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 540–546
34. Baldwin, A. S., Jr (1996) Annu. Rev. Immunol. 14, 649–683
35. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735
36. Canty, T. G., Boyle, E. M., Angie Farr, B. S., Morgan, E. N., Perlmutter, R. M., and Pohlman, T. H. (1999) Circulation 100, 361–364
37. Inhiber, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traneckner, E. B., Mueller-Diekemnann, C., Farahifar, D., Ross, B., and Engelhardt, J. F. (2003) J. Biol. Chem. 278, 2072–2080
38. Das, R., Mahabaleshwar, G. H., and Kundu, G. C. (2003) J. Biol. Chem. 278, 26593–26606
39. Philip, S., Bubule, A., and Kundu, G. C. (2001) J. Biol. Chem. 276, 44926–44935
40. Philip, S., and Kundu, G. C. (2003) J. Biol. Chem. 278, 14447–14447
41. Mahabaleshwar, G. H., and Kundu, G. C. (2003) J. Biol. Chem. 278, 6209–6221
42. Abraham, K. M., Levin, S. D., Marth, J. D., Forbush, K. A., and Perlmutt, R. M. (1991) Proc. Natl. Acad. Sci., U. S. A. 88, 3977–3981
43. Imbert, V., Farahifar, D., Auberger, P., and Engerhardt, J. F. (2003) J. Biol. Chem. 278, 276, 278, 278
44. Philip, S., and Kundu, G. C. (2003) J. Biol. Chem. 278, 14447–14447
45. Beraud, C, Henzel, W. J., and Baeuerle, P. A. (1999) Proc. Natl. Acad. Sci., U. S. A. 96, 429–444