Journal of Cellular Biochemistry 98:1570–1583 (2006)

Nuclear-Cytoplasmic Transport of EGFR Involves Receptor Endocytosis, Importin β1 and CRM1

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Abstract Many receptor tyrosine kinases (RTKs) can be detected in the cell nucleus, such as EGFR, HER-2, HER-3, HER-4, and fibroblast growth factor receptor. EGFR, HER-2 and HER-4 contain transactivational activity and function as transcription co-factors to activate gene promoters. High EGFR in tumor nuclei correlates with increased tumor proliferation and poor survival in cancer patients. However, the mechanism by which cell-surface EGFR translocates into the cell nucleus remains largely unknown. Here, we found that EGFR co-localizes and interacts with importins α1/β1, carriers that are critical for macromolecules nuclear import. EGFR variant mutated at the nuclear localization signal (NLS) is defective in associating with importins and in entering the nuclei indicating that EGFR's NLS is critical for EGFR/importins interaction and EGFR nuclear import. Moreover, disruption of receptor internalization process using chemicals and forced expression of dominant-negative Dynamin II mutant suppressed nuclear entry of EGFR. Additional evidences suggest an involvement of endosomal sorting machinery in EGFR nuclear translocalization. Finally, we found that nuclear export of EGFR may involve CRM1 exportin as we detected EGFR/CRM1 interaction and markedly increased nuclear EGFR following exposure to leptomycin B, a CRM1 inhibitor. Collectively, these data suggest the importance of receptor endocytosis, endosomal sorting machinery, interaction with importins α1/β1, and exportin CRM1 in EGFR nuclear-cytoplasmic trafficking. Together, our work sheds light into the nature and regulation of the nuclear EGFR pathway and provides a plausible mechanism by which cells shuttle cell-surface EGFR and potentially other RTKs through the nuclear pore complex and into the nuclear compartment. J. Cell. Biochem. 98: 1570–1583, 2006.

Key words: EGF receptor; importin β1; endocytosis; endosomal sorting; CRM1; cancer

Emerging evidences suggest a novel mode of EGF signaling in which growth signals can be directly transmitted to the nucleus, via EGFR nuclear transport [Marti et al., 1991; Lin et al., 2001; Bourguignon et al., 2002; Wells and Marti, 2002; Carpenter, 2003; Krolewski, 2005; Lo and Hung, 2006]. This direct route of EGFR signaling is distinct from the classical pathway which involves activation of multiple cascades [Cohen et al., 1982; Anderson et al., 1990; Yarden, 2001]. Other receptors in the ErbB family, including neu/HER-2, HER-3, truncated ErbB-4/HER-4, also exist in the cell nucleus [Xie and Hung, 1994; Marti and Hug, 1995; Ni et al., 2001; Offterdinger et al., 2002; Wang et al., 2004]. ErbB4 undergoes γ-secretase-mediated cleavage and the C-terminal 80-kDa fragment translocates into the cell nucleus [Ni et al., 2001; Carpenter, 2003; Cheng et al., 2003; Ni et al., 2003; Williams et al., 2004].

Other receptor tyrosine kinases (RTKs), including TrkA/NGFR, fibroblast growth factor

Hui-Wen Lo and Mohamed Ali-Seyed have contributed equally to this work.

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: National Institute of Health; Grant numbers: RO-1 CA109311, PO-1 CA99031, P50 83639, P20 CA101936 (70 M-C.H.); Grant sponsor: American Cancer Society; Grant number: PF-05-175-01-TBE (to H-W.L.); Grant sponsor: National Institute of Health; Grant number: CA16672.

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Received 5 December 2005; Accepted 7 February 2006
DOI 10.1002/jcb.20876

receptor (FGFR), insulin receptor, VEGF receptor 2, and TGF-β type I receptor also undergo nuclear transport [Podlecki et al., 1987; Rakowicz-Szulczynska et al., 1988; Maher, 1996; Stachowiak et al., 1997; Zwaagstra et al., 2000; Reilly and Maher, 2001; Zhang et al., 2003; Raabe et al., 2004; Pillai et al., 2005]. Receptors to inflammatory cytokines, such as, interleukin-1 (IL-1), IL-5, and interferon-γ (IFN-γ) also exist in the nuclear compartment [Curtis et al., 1990; Bader and Weitzerbin, 1994; Jans et al., 1997; Jans and Hassan, 1998; Larkin et al., 2000; Subramaniam et al., 2001; Subramaniam and Johnson, 2002, 2004]. Ligands to most of these RTKs and cytokine receptors, including EGF, NGF, FGF, IL-1, IL-5, TGF-α and -β, and IFN-γ were also found in the nucleus [Curtis et al., 1990; Jans and Hassan, 1998; Grasl-Kraupp et al., 2002].

While the nuclear function of these receptors remains unclear, a role in growth stimulation is suggested. EGFR and HER-2 interact with specific DNA sequences on the promoters of cyclin D1/inducible nitric oxide synthase and cyclooxygenase-2, respectively, leading to gene activation [Lin et al., 2001; Wang et al., 2004; Lo et al., 2005a]. Products of these genes are involved in tumorigenesis and tumor progression [Hunter and Pines, 1994; Oshima et al., 1996; Thomsen et al., 1997]. More recently, nuclear EGFR and E2F1 interact with B-Myb promoter and activate its transcription, leading to accelerated G1/S cell cycle progression [Hanada et al., 2006]. In primary breast carcinomas, nuclear EGFR positively correlates with high proliferative potentials as indicated by increased expression of cyclin D1 and Ki-67 [Lo et al., 2005b]. Accumulation of EGFR in tumor nuclei correlates with poor survival in patients with breast cancer and oropharyngeal carcinomas [Lo et al., 2005b; Psyrri et al., 2005]. Consistently, EGFR undergoes nuclear translocalization in regenerating hepatocytes [Marti et al., 1991; Marti and Hug, 1995; Schausberger et al., 2003]. Similarly, nuclear import of FGFR associates with proliferation [Stachowiak et al., 1997].

Interestingly, some of these receptors exist in the nucleus as intact proteins including EGFR, HER-2, HER-3, and FGFR [Stachowiak et al., 1997; Lin et al., 2001; Offterdinger et al., 2002; Wang et al., 2004]. However, the mechanism for nuclear transport of intact receptors is elusive. Nevertheless, ligands activate the nuclear import of EGFR and FGFR [Lin et al., 2001; Reilly and Maher, 2001]. A possibility is thus raised that ligand-induced receptor internalization may involve in EGFR nuclear transport.

Transport of proteins through the nuclear-pore-complex can involve transport receptors, importins α/β and exportins [Gorlich and Kutay, 1999]. Specifically, importin-α binds to the classical lysine-rich nuclear localization signal (NLS) in the cargo and importin-β interacts with importin-α/cargo complex guiding them through the nuclear pore. However, evidences suggest that nuclear import can occur via an interaction of non-classical arginine-rich NLS and importin-β [Cingolani et al., 2002]. Indeed, an arginine-rich putative NLS is present within EGFR and required for its nuclear entry [Lin et al., 2001; Lo et al., 2005a]. Association of importin β1 has been shown to be required for nuclear translocalization of both FGFR and HER-2 [Reilly and Maher, 2001; Giri et al., 2005]. It is thus possible that EGFR enters the nucleus via a mechanism that involves EGFR putative NLS and importin β1. Collectively the current study describes two major events occurring during the transit of EGFR from the cell-surface into the nucleus, namely, receptor internalization and EGFR/importin β1 interaction. These findings shed light into the mechanism underlying EGFR nuclear transport, which can be utilized by other cell-surface receptors that also undergo nuclear-cytoplasmic shuttling.

RESULTS AND DISCUSSION

EGFR Interacts With Importin β1

As importin β1 plays a critical role in nuclear import, we asked whether EGFR nuclear entry involved importin β1. We found that EGFR interacts with importin β1 and the interaction enhanced by EGF as indicated by immunoprecipitation/western blots, IP/WB, shown in Figure 1A. Using electron microscopy (EM) analysis, we observed increased EGFR/importin β1 co-localization following EGF treatment (Fig. 1B). Inset a shows a representative image of the unbound EGFR in untreated MDA-MB-468 cells (upper panel, Fig. 1B). In contrast, insets b&c (middle panel) represent importin β1-bound EGFR in EGF-treated cancer cells in which some EGFR co-localized with importin β1 within the nucleus (inset b) and in the vicinity of nuclear membranes (inset c). Bottom panel

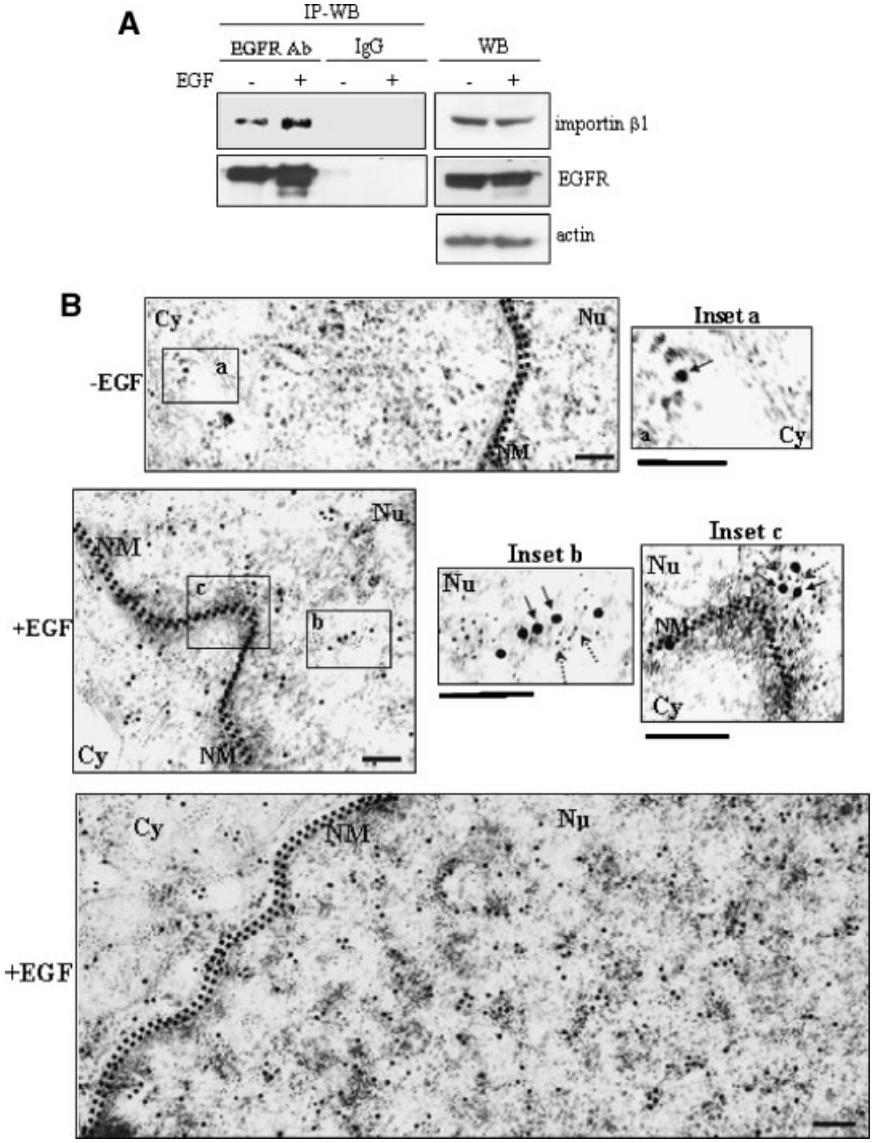

Fig. 1. Interaction of EGFR and importin β1. **A**: Association of EGFR and importin β1. A431 whole cell lysates were subjected to co-IP analysis using EGFR Ab or IgG, followed by WB analysis using importin β1 and EGFR Abs (**left**). Endogenous levels of importin β1 and EGFR were simultaneously determined by WB analyses in which β-actin was also detected to serve as loading controls (**right**). **B**, **C**: Co-localization of EGFR and importin β1 by EM (B) and IF/confocal (C) analyses. MDA-MB-468 cells were treated with and without EGF (100 ng/ml) for 30 min. In (B), secondary Abs labeled with 15-nm and 5-nm gold particles were used to indicate EGFR and importin β1, respectively. Solid arrows mark EGFR and dashed arrows indicate importin β1. Cy, cytosol; Nu, nucleus; NM, nuclear membrane marked by dashed lines; Bar, 100 nm. In the EM studies (B), the 15-nm gold particles are capable of absorbing 7.8 IgG molecules per particle. With this regard, each gold particle represents 1–7 molecules of primary/secondary antibody and 1–14 EGFR molecules. In (C), cells were incubated with EGF for 30 min and immuno-stained for EGFR (red) and importin β1 (green). Nuclei are stained by ToPro3 and indicated as blue. Arrows mark yellow signals that indicate co-localized EGFR/importin β1. All insets demonstrate enlarged high-resolution images. **D**: Nuclear EGFR did not co-localize with the ER marker calregulin. MDA-MB-468 cells treated as in (C) were immuno-stained for EGFR (red) and calregulin (green) and then subjected to IF/confocal analyses. Arrows mark nuclear EGFR (red) that did not co-localize with calregulin. **E**: Reduced interaction of EGFR-pNLS mutant with importin β1. Association of EGFR proteins with importin β1 was analyzed, via co-IP/WB, in transiently transfected MDA-MB-231 cells (**left**). Binding affinity is expressed as importin β1/EGFR. As these cells express low levels of endogenous EGFR, importin β1/EGFR ratio in pNEO-transfected cells is expressed as 1.0. **Right**: WB analysis.

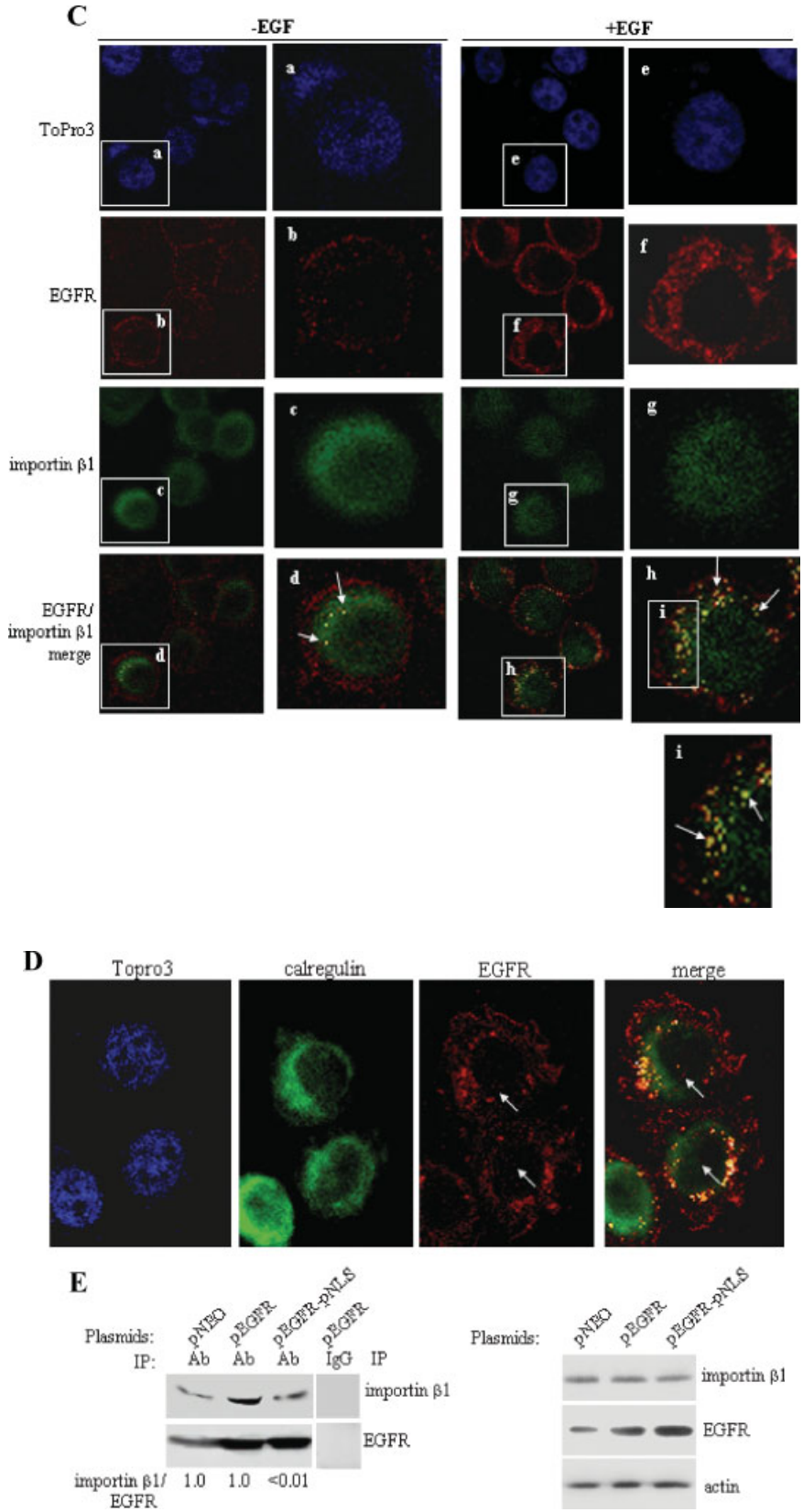

Fig. 1. (*Continued*)

further shows significant co-localization of nuclear EGFR/importin β1 and high levels of EGFR nuclear transport after EGF stimulation. The observed co-localization pattern resembles those reported previously [Violot et al., 2003]. EGF-induced EGFR/importin β1 interaction was further confirmed by immunofluorescence (IF)/confocal analyses (Fig. 1C). Importantly, nuclear EGFR did not co-localize with the ER marker, calregulin (Fig. 1D), indicating EGFR was indeed in the nucleus but not on the ER membrane that is contiguous with the nuclear membrane. Together, these results indicate that EGFR co-localizes and interacts with the nuclear transport protein, importin β1, and the co-localization/interaction was enhanced by EGF treatment.

In line with the observations, importin β1 association is critical for FGFR and HER-2 nuclear import [Reilly and Maher, 2001; Giri et al., 2005]. Our data are also supported by previous reports showing that the interaction between non-classical NLS-containing cargos and importin β is crucial for the nuclear import. These cargos include cdc25C [Schwindling et al., 2004], parathyroid hormone-related protein [Cingolani et al., 2002], Rex protein of human T-cell leukemia virus type 1 [Palmeri and Malim, 1999], and Tat and Rev from HIV type I [Truant and Cullen, 1999].

EGFR NLS Is Important for EGFR/Importin β1 Interaction

A putative pNLS is contained in EGFR which directs β-galactosidase to the nucleus and important for EGFR nuclear entry [Lin et al., 2001; Lo et al., 2005a]. As importins/NLS interaction is required for nuclear entry of many cargos and EGFR interacts with importin β1 (Fig. 1A), we asked whether EGFR pNLS mutant is defective in importin β1 interaction and therefore fails to enter the nucleus. We transiently transfected the parental vector and those with wild-type and mutant EGFR into MDA-MB-231 cells who express low endogenous levels of EGFR. The interaction between importin β1 and EGFR was then examined by co-IP/WB analyses (Fig. 1E, left). The degree of the association between importin β1 and EGFR is expressed as importin β1:EGFR, with that from the pNEO transfectants as 1.0. The transfected wild-type EGFR (lane 2) associated with importin β1 equally well as the endogenous EGFR (lane 1). However, the association of transfected EGFR-pNLS mutant (lane 3) with importin β1 was significantly reduced in comparison with the endogenous EGFR (lane 1) and transfected wild-type EGFR (lane 2), as the intensity of importin β1 Ab in cells transfected with the EGFR-pNLS mutant (lane 3) did not increase as in those transfected with the wild-type EGFR (lane 2). Equal amount of cell lysates were loaded and that tumors cells were efficiently transfected to express EGFR (Fig. 1E, right). Our data here are in line with our previous study showing that HER-2 mutated at the NLS region failed to associate with importin β1 and, therefore, was unable to enter the cell nucleus [Giri et al., 2005]. Consistent with these observations, EGFR-pNLS mutant did not significantly increase cyclin D1 promoter (Fig. S1A) and expression (Fig. S1B), following EGF stimulation, in which cyclin D1 gene activity was regarded as a measure for nuclear EGFR biological activity.

We also observed co-localization of EGFR and importin α1 near the cell surface (upper left) and in the cytosol and nucleus (upper right) as indicated by EM (Fig. 2A) and IF/confocal analyses (Fig. 2B). A larger view of nuclear compartment is shown in Figure 2A (lower panel) which demonstrates significant co-localization of EGFR and importin α1 in the nucleus. This is consistent with a previous report showing that EGFR interacts with importin α1 by co-IP/WB method [Dittmann et al., 2005]. Together, these results indicate that EGFR interacts with nuclear transport receptors, importin α1 and importin β1 and that the EGFR-pNLS is required for EGFR/importin β1 interaction and for the subsequent nuclear import.

Endocytosis Inhibition Reduces EGF-Activated EGFR Nuclear Transport

We have previously shown that EGF activates nuclear transport of full-length EGFR in MDA-MB-468 human breast carcinoma cells using IF/confocal and WB methods [Lin et al., 2001; Bourguignon et al., 2002; Lo et al., 2005b]. Here, we further demonstrate via EM method that EGF activates EGFR nuclear import. EGF-treated MDA-MB-468 cells contained significantly more EGFR in the nucleus compared to the untreated cells (upper panel in Fig. 3A and Fig. S2 in the Supplemental Data). Consistent with the EM observation, IF/confocal analysis (lower panel in Fig. 3A) shows increased nuclear

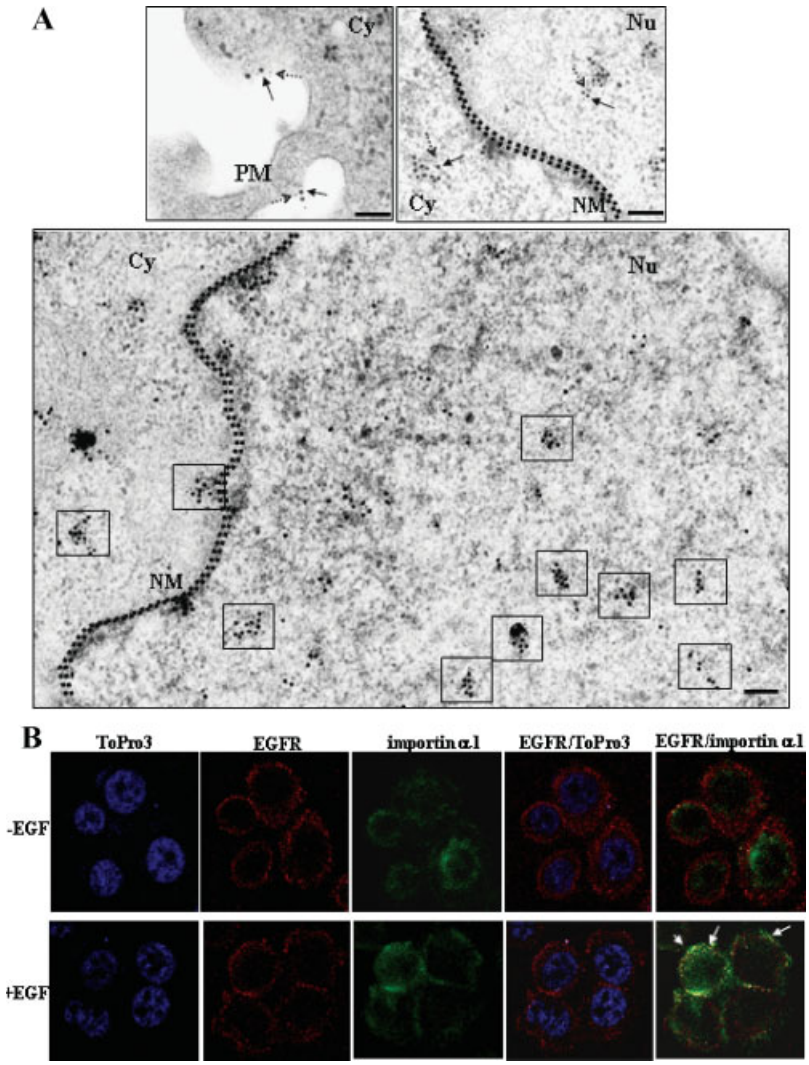

Fig. 2. EGFR co-localizes with importin α1. Co-localization of EGFR/importin α1 was analyzed by EM (**A**) and IF/confocal analyses (**B**). MDA-MB-468 cells were treated and analyzed as previously described in Figure 1, except that the importin α1 Ab replaced importinβ1 Ab. In EM (A), cells were treated with EGF and solid arrows indicate EGFR (15 nm gold particles) whereas dashed arrows point to importin α1 (5-nm particles). In the **bottom panel**, multiple EGFR/importin α1 complexes (marked by boxed) were observed in a larger field. Cy, cytosol; Nu, nucleus; NM, nuclear membrane; PM, plasma membrane; Bar, 100-nm. In (B), cells treated without and with EGF were subjected to IF/confocal analyses. Arrows mark yellow signals that indicate co-localized EGFR (red) and importin α1 (green).

EGFR (merged pink signals) after EGF stimulation. Similar to EGFR, IFN-γ activates IFNγR-1 nuclear transport 10–20 min post-treatment [Larkin et al., 2000]. Heregulin activates perinuclear accumulation of HER-2 [Bacus et al., 1992]. FGF-2, NGF, IL-1 and IL-5 result in nuclear import of their receptors [Curtis et al., 1990; Jans et al., 1997; Reilly and Maher, 2001; Zhang et al., 2003].

As EGF activates EGFR nuclear import, we speculated that EGF-mediated internalization might be required for cell-surface EGFR to enter the nucleus. It is known that EGF-bound EGFR is rapidly internalized via clatherin-coated pits into early endosomes [Haigler et al., 1979] in which Eps15 (EGFR pathway substrate clone 15) and dynamin are important for the formation of clatherin-coated pits [Damke et al., 1994]. Additionally, dynamin is also involved in caveolae-dependent endocytosis [Henley et al., 1998]. To this end, we reasoned that expression of defective dynamin would inhibit EGFR nuclear transport if the process requires endocytosis. Indeed, transient expression of the

dynamin II/K44A mutant in EGF-treated CHO-EGFR cells reduced nuclear EGFR levels by approximately 80% (Fig. 3B).

Consistently, pre-treatment using the endocytosis inhibitor phenylarsine oxide (PAO) significantly blocked EGF-activated nuclear import of EGFR (Fig. 3C). Lack of CD44, a cell-surface protein, and α-tubulin, a cytoplasmic protein, in the nuclear lysates indicates efficient cell fractionation. Inhibition of EGFR kinase activity, a property important for its internalization [Chen et al., 1987], by the inhibitor PD158780 also reduced the levels of EGFR in the nucleus. Similar observations were found when tumors cells were subjected to IF/deconvolution analysis in which only 14% and 3% of

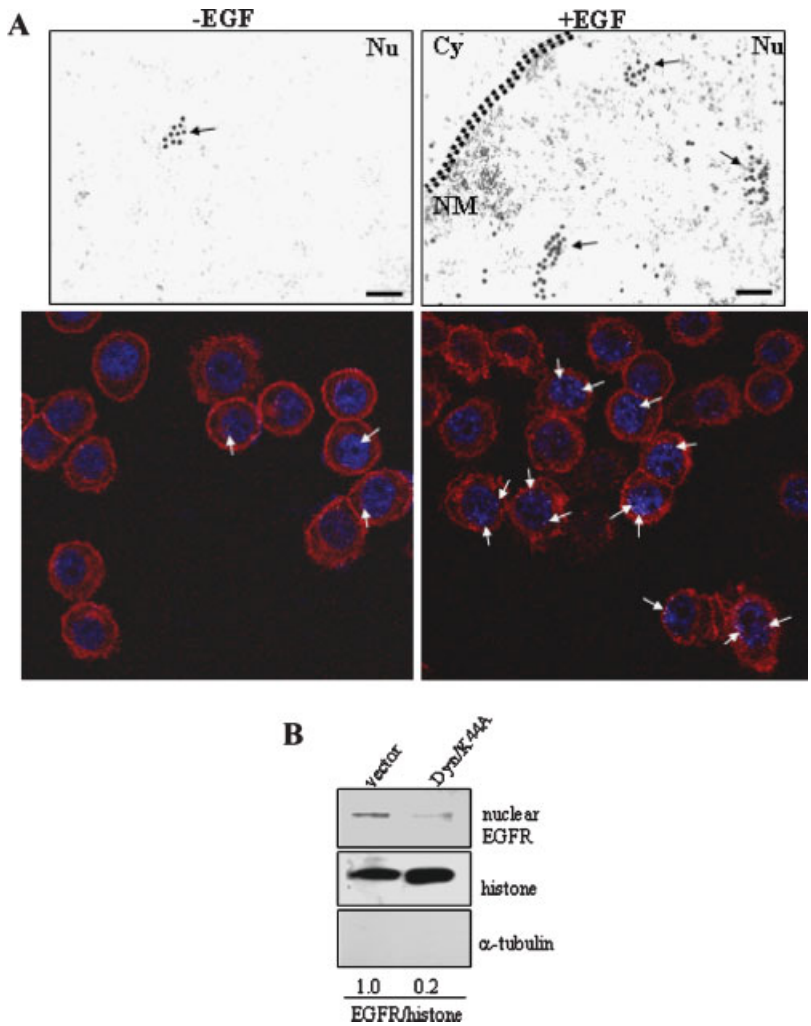

Fig. 3. Endocytosis inhibition reduces EGF-activated EGFR nuclear transport. **A**: EGF activated EGFR nuclear import as shown by EM. MDA-MB-468 cells were serum-starved for 24-h, treated without (-EGF) and with EGF for 30 min and subjected to EM (**top**) and IF/confocal analysis (**bottom**). Arrows point to nuclear EGFR. Cy, cytosol; Nu, nucleus; NM, nuclear membrane indicated by dashed lines; Bar, 100 nm. In IF/confocal analysis (bottom), EGFR is indicated by the red signals and the nuclei, by the blue. Arrows mark pink signals that indicate nuclear EGFR (merge of red and blue). **B**: Inhibition of EGFR nuclear transport by dominant-negative dynamin II mutant. CHO-EGFR cells transiently transfected with indicated vectors were stimulated with EGF and nuclear EGFR detected by WB method. Nuclear EGFR in each reaction was normalized with those of histone H3A and expressed relative to the vector control (1.0). **C**, **D**: Reduction of EGFR nuclear transport by endocytosis inhibitor. A431 cells pre-treated for 1-h with indicated inhibitors, PAO or PD158780, were stimulated with EGF, and levels of EGFR determined by WB method (C) and IF/deconvolution analysis (D). Histone H3A and CD44 serve as markers for nuclear and plasma membrane proteins, respectively. β-actin and α-tubulin were also detected and considered as cytosolic markers. Relative nuclear EGFR levels were calculated as ratios of nuclear EGFR/histone H3A. In IF/deconvolution analysis (D), only EGF-stimulated cells were immunostained in which EGFR is indicated by the green signals and the nuclei, by the red. Yellow signals represent nuclear EGFR (merge of green/EGFR and red/nuclei). Percentage of tumor cells stained positive for nuclear EGFR was calculated from three independent experiments and in each experiment, a total of 150–200 cells were evaluated.

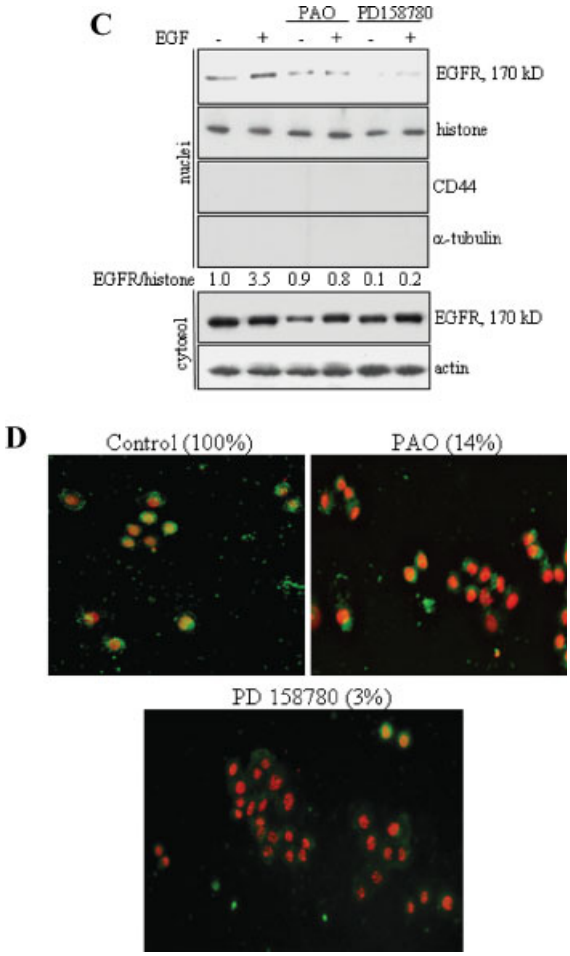

Fig. 3. (*Continued*)

PAO- and PD 158780-treated cells contained nuclear EGFR (Fig. 3D). In these studies, percentage of cells with nuclear EGFR was calculated from three independent experiments and a total of 150–200 cells were evaluated per each experiment. Consistent with our findings, IFNγR-1 has been shown to undergo clatherin- and caveolae-mediated endocytosis upon IFN-γ binding and such internalization is required for nuclear entry of IFN-γ and IFNγR-1 [Subramaniam and Johnson, 2002]. Transient expression of the dynamin II/K44A mutant significantly reduced HER-2 nuclear transport [Giri et al., 2005]. Together, these findings suggest that receptor internalization may serve as an important initial step for nuclear entry of cell-surface receptors including EGFR.

Endosomal Sorting Is Involved in EGFR Nuclear Transport

Internalized EGFR can be recycled back to the cell-surface or sorted into late endosomes and then ultimately degraded by lysosomes [Sorkin and Von Zastrow, 2002]. However, the rate of EGFR internalization is much faster than that of degradation [Wiley et al., 1985], suggesting a substantial amount of EGFR can accumulate in the cytoplasm and may potentially escape lysosome-mediated degradation. Thus, we rationalized that endocytic vesicles may serve as carriers that shuttle EGFR into the cell nucleus. Here, we observed co-localization of EGFR and early endosome antigen-1 (EEA1, an early endosome maker) in EGF-treated MDA-MB-468 cells (Fig. 4A). EGFR/

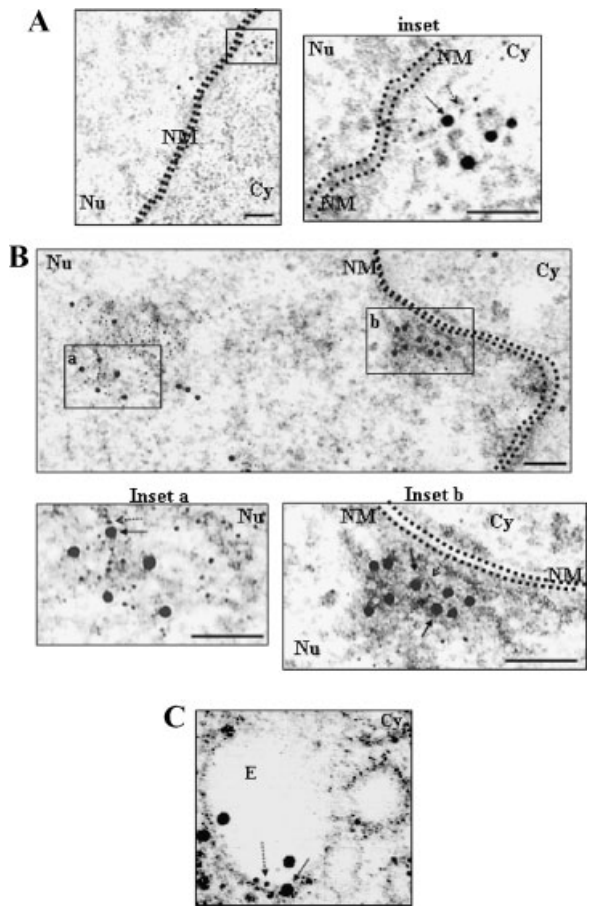

Fig. 4. Endosomal sorting may be involved in EGFR nuclear transport. Serum-starved MDA-MB-468 cells were treated with EGF (100 ng/ml) for 30 min and subjected to EM analysis. Cy, cytosol; Nu, nucleus; NM, nuclear membrane marked by dashed lines; Bar, 100 nm. **A**: Peri-nuclear co-localization of EGFR and early endosome marker EEA1. EGFR (15-nm gold particles) and EEA1 (5-nm gold particles) were indicated by solid and dashed arrows, respectively. **B**: EGFR co-localized with EEA1 in the nucleus (inset a) and near the inner nuclear membrane (inset b). **C**: EGFR/importin β1 co-localization on endocytic vesicles. Solid arrows indicate EGFR (15-nm gold particles) and the dashed arrows label importin β1 (5-nm gold particles); (E), endocytic vesicle.

EEA1 co-localization occurs in the cytosol as expected (data not shown) and in vicinity of the nuclear envelope (Fig. 4A) which resembles association of HER-2 and EEA1 [Giri et al., 2005]. Furthermore, we also observed co-localization within the nuclear compartment (inset a) and near the inner nuclear membrane (inset b, Fig. 4B) which is in agreement with our previous report showing EEA1 undergoes nuclear transport via an importin β1-mediated mechanism [Giri et al., 2005]. Interestingly, we detected co-localization of EGFR and importin β1 on the endocytic vesicles in EGF-treated cells (Fig. 4C), suggesting a role for endosomes in nuclear shutting of EGFR. Together, these observations suggest a possible involvement of endosomal sorting in EGFR nuclear translocalization.

Consistent with our findings, Bild et al. [2002] showed that endosomal EGF co-transits with STAT3, a nuclear protein associating with activated EGFR near the inner surface of cytoplasmic membrane, from the cell-surface to the peri-nuclear region. Inhibition of receptor endocytosis, chemically and genetically, led to blockage of STAT3 nuclear activity as measured by its DNA binding and transactivational abilities [Bild et al., 2002]. TGF-β receptor internalization into EEA1-enriched early endosomes is required for Smad2 nuclear translocalization and transcriptional activation of TGFβ-responsive genes [Hayes et al., 2002]. More recently, Miaczynska et al. [2004] reported that association of APPLs and Rad5 on endosomes, a small GTPase that is important for the formation of early endosomes, leads to nuclear localization of APPLs. In the nucleus, APPL1 and APPL2 are important for cell proliferation through their interaction with chromatin remodeling complex, NuRD/MeCP. β-arrestins, proteins involved in endocytosis of G protein-coupled receptors, also enter the nuclei and regulates histone acetylation and gene expression [Kang et al., 2005]. Most recently, a potential involvement of endosomal sorting in HER-2 nuclear entry has been suggested [Giri et al., 2005].

Kinetic Interaction Between EGFR and Transport Receptors, Importin β1 and CRM1

Transport of nuclear proteins through the nuclear-pore-complex often requires transport receptors, importins α/β and chromatin region maintenance, CRM1 [Gorlich and Kutay, 1999]. Our recent report shows that nuclear export of HER-2 involves its physical association with nuclear export receptor CRM1 [Giri et al., 2005]. To elucidate the mechanism by which nuclear EGFR exits the nuclear compartment, we thus examined the involvement of CRM1. As indicated in Figure 5A, CRM1 co-immunoprecipitated with EGFR following EGF stimulation. EGFR/CRM1 interaction increased time-dependently with the strongest interaction at 45-min post-treatment. In contrast, IgG did not precipitate CRM1 or EGFR indicating specificity. Furthermore, reverse IP using EGFR Ab detected consistent EGF-dependent interactions between EGFR and CRM1 (Fig. 5B). A similar EGFR/CRM1 association kinetics was observed, in which the interaction increased in

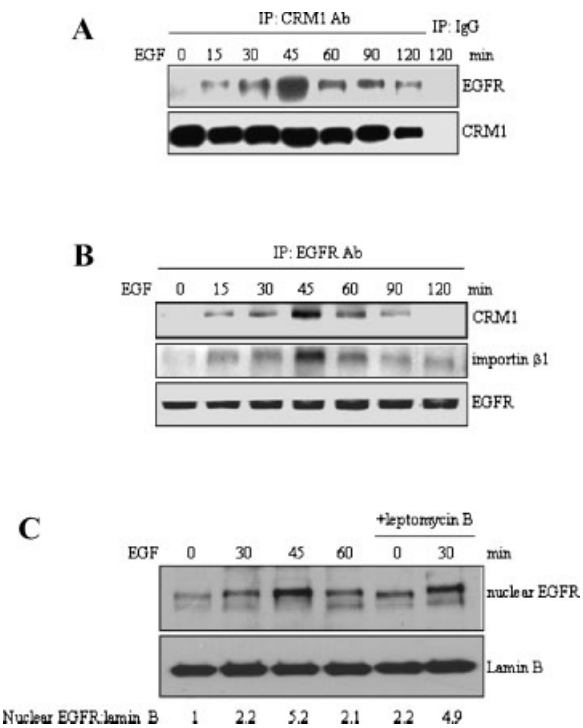

Fig. 5. Time-dependent interaction of EGFR and transport receptors, CRM1 and importin β1. **A**, **B**: Serum-starved A431 cells were treated with EGF (100 ng/ml) for 0, 15, 30, 45, 60, 90, and 120 min, harvested and then whole cell lysates were extracted. A431 whole cell lysates were subjected to co-IP analysis using CRM1 Ab (A) and EGFR Ab (B), followed by WB analysis using CRM1, importin β1, and EGFR Abs. **C**: A431 cells were serum-starved and then treated with EGF (100 ng/ml) for 0, 30, 45, and 60 min. Harvested cells were then fractionated and nuclear lysates were analyzed by WB for the levels of nuclear EGFR and the nuclear marker, lamin B. Simultaneously, aliquots of A431 cells were pre-treated with 20 ng/ml leptomycin B for 4 h and stimulated with EGF (100 ng/ml) for 0 and 30 min. Nuclear lysates were extracted and analyzed for nuclear EGFR expression.

a time-dependent fashion up to 45 min following EGF stimulation and then declined time-dependently to the basal level at 120 min. EGFR associates with nuclear import receptor, importin β1, in a pattern that is similar to the interaction of EGFR/CRM1. Consistently, nuclear translocalization of EGFR peaked at 45 min (5.2-fold) after EGF stimulation and declined at 1-h post-treatment (Fig. 5C). Finally, nuclear EGFR levels were enhanced by pre-treatment of CRM1 inhibitor, leptomycin B, indicating the involvement of exportin CRM1 in EGFR nuclear export. These results suggest the involvement of both importin β1 and CRM1 in the nuclear-cytoplasmic transport of EGFR.

A detailed molecular event required for EGFR nuclear entry still awaits investigation. Given that endosomes have never been detected in the nucleus, a yet un-identified mechanism must exist to remove EGFR from endosomal membrane prior to its entry to the nucleus. In a hypothetical model by Carpenter [2003], EGFR-bearing endosomes first fuse with Golgi, retrograded into ER and extracted by an ER-associated degradation (ERAD) system, a known system that extracts mal-folded proteins in the ER into the cytoplasm. Through such system, EGFR can be released into cytoplasm existing in a non-membrane-bound form and then subsequently interacts with nuclear pore complex [Giri et al., 2005] for nuclear entry like HER-2. Whether endosomal EGFR indeed utilizes this ERAD system to escape from endosomes requires future extensive investigation.

In summary, this study reports several key findings that shed light into the mechanisms underlying EGFR nuclear import. First, we demonstrated that both EGF stimulation and receptor internalization serve as an initial step for EGFR nuclear entry. Second, we have evidences showing that EGFR physically interacts with importin β1 via its pNLS region and that this interaction is required for EGFR nuclear import. Third, our data suggest a potential involvement of endosomal sorting machinery in EGFR nuclear transport. Finally, we found CRM to involve in EGFR nucear export. We believe that these findings contribute significantly to a better understanding of intracellular trafficking of not only EGFR but also other RTKs that shuttle between the cell-surface and nucleus.

METHODS

Cell Lines and Cell Culture

MDA-MB-468 and MDA-MB-231 human breast carcinoma cells, A431 human epidermoid carcinoma cells, and Chinese hamster ovary (CHO) cells were obtained from ATCC. CHO stable cells, CHO-NEO, CHO-EGFR and CHO-EGFR-pNLS, were derived from parental EGFR-null CHO cells [Lo et al., 2005a]. All cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Stable CHO lines were additionally supplemented with G418.

Chemicals, Plasmids, and Mammalian Transfection

PD158780 was obtained from Calbiochem (San Diego, CA) and all other chemicals and purified EGF were from Sigma-Aldrich (St. Louis, MO). EGFR expression plasmid was a gift from Dr. David James at Mayo Clinic whereas the parental pcDNA3.1 vector was obtained from Invitrogen (Carlsbad, CA). Expression plasmids containing GFP-tagged dynamin II, pGFP-Dyn, and pGFP-Dyn/K44A were kind gifts from Dr. Mark McNiven at Mayo Clinic whereas the parental plasmid pEGFP-N1 was purchased from Clonetech (Palo Alto, CA). Transfection was carried out by an electroporation method using Nucleofactor (Amaxa, Nattermannallee, Germany) or by a cationic liposome, SN, as previously described [Zou et al., 2002].

Immunoprecipitation and WB Analyses

For IP experiments, cells treated per experimental procedures were washed, nuclear fractions extracted, and pre-cleared with 0.8 μg rabbit normal serum and 20 μl protein A-agarose (Santa Cruz) for 1.5 h at 4°C. Pre-cleared lysates were then incubated with 1 μg monoclonal anti-EGFR Ab (Ab-13, Neomarkers), rabbit polyclonal CRM1 Ab (H-300, Santa Cruz Biotech.) or normal serum at 4°C overnight with gentle agitation. Following addition of protein G-agarose, incubation was continued for additional 30 min at 4°C. Protein G-agarose pellets were collected and washed for multiple cycles at 4°C. The washed immunoprecipitates were subjected to SDS–PAGE and WB analysis. The antibodies used for WB analyses included rabbit polyclonal EGFR (sc-03), CRM1 (H-300), importin β1 (H-300) antibodies from Santa

Cruz Biotech., monoclonal β-actin and α-tubulin antibodies (Sigma-Aldrich), polyclonal rabbit histone H3A and cyclin D1 antibodies from Cell Signaling (Beverly, MA), and monoclonal CD44 antibody (Neomarkers). Monoclonal mouse anti-lamin B antibody (101-B7) was purchase from Calbiochem.

Transmission EM

MDA-MB-468 cells per experimental procedures were harvested, washed with PBS, syringed to get single cell suspension, and subjected to EM analysis as described previously [Wang et al., 2004]. Following incubation with either mouse or rabbit isotypic control immunoglobulins, sections were treated with specific primary Abs and then with gold particle (5 or 15 nm) labeled with either goat anti-mouse or anti-rabbit secondary Ab (Amersham Biosciences). Sections were washed, stained with uranyl acetate and Reynolds's lead citrate and examined in a Jeol 1200EX microscope. In the EM studies, the 15-nm gold particles are capable of absorbing 7.8 IgG molecules per particle. With this regard, each gold particle represents 1–7 molecules of primary/secondary antibody and 1–14 EGFR molecules. Primary Abs used include those against EGFR (1:300, Zymed and Novocastra), importin α1 (goat Ab, Santa Cruz), importin β1 (1:200, Santa Cruz), and EEA1 (1:200, Upstate).

Immunofluorescent Microscopy and Confocal Analyses

In the analyses of the kinetics of EGFR endocytosis, serum-starved cells grown on Lab-tek chamber slides (2×10^4 cells/ml) were incubated at 4°C with and without 100 ng/ml EGF for 1 h and moved to 37°C medium, to initiate endocytosis, for 30 min. The inhibitor studies were performed at 37°C in which cells were pre-treated with 5 μM PAO or 5 μM PD158780 for 1 h prior to 30 min EGF stimulation. PAO has been shown to efficiently block endocytosis [Hertel et al., 1985] whereas PD158780 is a EGFR kinase inhibitor [Rewcastle et al., 1998]. Cells were then washed three times with ice-cold PBS, fixed in 4% paraformaldehyde for 15 min, and permeablized using 0.2% Triton X-100. Following treatment with 0.1% normal goat serum or isotypic IgG for 30 min, cells were incubated with indicated primary Abs for 1 h, washed and further incubated with respective secondary antibody (Vector) tagged with either FITC or with Texas Red diluted at 1:300. Primary antibodies used in these studies include rabbit polyclonal EGFR antibody (1:300, Santa Cruz Biotech.), and monoclonal EGFR antibodies (1:300, Zymed and Novocastra), rabbit calregulin (1:200, Santa Cruz), rabbit polyclonal importin β1 (1:200, Santa Cruz), and goat polyclonal importin α1 (Santa Cruz) antibodies. To delineate the nuclear morphology, nuclear marker ToPro3 was used. The green and red fluorescence of FITC and TexaRed, respectively, were visualized and images were captured using a Zeiss AxioPlan2 (Germany) equipped with Carl Zeiss/CSM510 for confocal analyses.

Cellular Fractionation

Cellular fractionation was performed as previously described [Dignam et al., 1983]. Cells treated per experimental procedures were collected, washed with PBS, and swelled in hypotonic buffer (25 mM Tris-HCl, pH 7.5, 5 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM PMSF and 0.15 μ/ml aprotinin) for 20 min on ice. Following homogenization using a Dounce homogenizer, nuclei were pelleted and washed. To extract nuclear proteins from the isolated nuclei, we used an ultrasonic disruption step as previously described by Wells et al. [2002] and sonication buffer containing 50 mM Tris-HCl 0.15 μ/ml aprotinin. Thereby, we extracted the soluble fraction of the nuclear preps leaving the membrane fractions insoluble. Since the EGFR remains membrane-bound on the endosomal membranes (early and late endosomes and multi-vesicular-bodies), these endosomal EGFR are thus in the insoluble fraction rather than in our nuclear extract. The supernatant was centrifuged at 15,000*g* to remove cell debris and the resulting supernatant was collected as the cytosolic fraction.

ACKNOWLEDGMENTS

This study is partially supported by RO-1 CA109311, PO-1 CA99031, the P50 83639, P20 CA101936 (to M-C.H.) and M. D. Anderson Cancer Center, Cancer Center Supporting Grant, CA16672 from the National Institute of Health. H-W.L. is a recipient of the Post-doctoral Fellowships from the American Cancer Society (PF-05-175-01-TBE to H-W.L.).

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