Tyrosine Phosphorylation of the β2 Subunit of Clathrin Adaptor Complex AP-2 Reveals the Role of a Di-leucine Motif in the Epidermal Growth Factor Receptor Trafficking

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Tyrosine phosphorylation of the β2 subunit of clathrin adaptor complex AP-2 was detected in three types of cells treated with epidermal growth factor (EGF). The tyrosine phosphorylation was observed during recruitment of EGF receptors into coated pits at 4 °C and reached maximum at 37 °C at post-recruitment stages of endocytosis. An inhibitor of EGF receptor kinase completely abolished this phosphorylation in all cell types, whereas the inhibitor of Src family kinases partially inhibited β2 phosphorylation in A-431 cells but not in HeLa cells. By using β2 subunit tagged with yellow fluorescent protein that is effectively assembled into AP-2 complex, the major phosphorylation site of β2 was mapped to Tyr-6. Analysis of cells expressing dominant-interfering mutant β2 subunit of AP-2 suggested that β2 phosphorylation is partially mediated by the receptor interaction with the μ2 subunit. Mutation of leucine residues 1010 and 1011 motif in the EGF receptor resulted in the severe inhibition of β2 tyrosine phosphorylation. From these data, we propose that interactions of the EGF receptor with AP-2 mediated by the receptor 97YRAL and di-leucine motifs may contribute to β2 tyrosine phosphorylation. Surprisingly, mutation of the Leu-1010/Leu-1011 motif resulted in impaired degradation of EGF receptors, suggesting the role of this motif in lysosomal targeting of the receptor.

Clathrin coats are specialized membrane structures that facilitate formation of endocytic vesicles and transport intermediates from the plasma membrane, Golgi complex, and possibly endosomes. Clathrin triskelions and clathrin adaptor complex AP-2 are the major components of the clathrin coats located at the plasma membrane, which are responsible for endocytosis of various proteins, lipids, and viral particles (1, 2). AP-2 is a stable tetramer consisting of the following four subunits: 100-kDa proteins α and β2, also called adaptins, μ2 (50 kDa), and σ2 (17 kDa) (3). Cryoelectron microscopy analysis suggested that AP-2 has so-called “trunk” or core domain that is composed of σ2, μ2, and amino-terminal parts of α and β2. Flexible hinge domains connect trunk with α and β2 “ear” domains (3). The crystal structures of the ears and the trunk domains of AP-2 have been solved recently (4).

AP-2 is engaged in interactions with multiple proteins and lipids. The main clathrin binding interface is located within the hinge domain of β2 (5), although α subunit can also interact with clathrin (6). The amino terminus of α subunit is capable of binding to phosphoinositides and inositol polyphosphates, and these interactions are thought to serve important roles in the regulation of membrane docking of AP-2 (7). The carboxyl-terminal domain of a subunit (α-ear) binds to several proteins containing DPF(W) motifs, such as Eps15, epsin, and CALM/AP180 (8). Individual subunits are engaged into multiple inter-subunit interactions within the trunk domain, which is necessary for the assembly of the AP-2 complex (4).

Besides the structural and scaffolding functions of AP-2 in coated pits, AP-2 is involved in the recruitment of endocytic cargo to clathrin coats by means of recognition of the specific internalization sequence motifs in the cytoplasmic tails of the cargo. The interaction of YXXφ motif with the μ2 subunit of AP-2 is best understood at the functional and structural levels (9). Di-leucine-containing motifs may interact with AP-2 either through μ2 (10) or β2 subunit (11). The interactions with AP-2 are critical for internalization of many types of cargo proteins because mutations in AP-2-binding motifs dramatically reduced their internalization rates (12). In contrast, despite that the interaction of the epidermal growth factor receptor (EGFR)1 with μ2/AP-2 was demonstrated by several techniques in vivo and in vitro, this interaction is not essential for EGFR internalization, and its involvement in EGFR endocytosis may be limited to certain experimental systems and cell types (13–15).

The mechanisms of regulation of multiple interactions of AP-2 with cargo, membrane bilayer, clathrin, and accessory proteins are not fully understood. Serine/threonine phosphorylation of a β subunit appears to regulate β2 interaction with clathrin triskelions (16). Recently, phosphorylation of μ2 was shown to play a role in the interaction of μ2/AP-2 with cargo (17). Binding of phospholipids and phosphoinositides has also been proposed to modify the cargo binding ability of AP-2 (18).

In this work we found a new modification of AP-2, a tyrosine phosphorylation of β2 subunit, in cells stimulated with EGF. Mutagenesis of ectopically expressed β2 fused to yellow fluorescent protein (YFP) revealed one phosphorylation site in the amino terminus of β2 that is partially responsible for this phosphorylation. Analysis of EGFR mutants exposed the im-

1 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; CMF-PBS, Ca2+-, Mg2+-free phosphate-buffered saline; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PAE, porcine aortic endothelial; HA, hemagglutinin; BSA, bovine serum albumin; Ab, antibody; DAPI, 4,6-diamidino-2-phenylindole.
portance of a di-leucine motif of the receptor for β2 phosphor-
ylation. This motif appears to be involved in the regulation of
the turnover of EGFR protein.

**Experimental Procedures**

Reagents—Mouse receptor-grade EGF was obtained from Collabora-
tive Research Inc. and iodinated using a modified chloramine-T method
as described previously (15). The specific activity of [125I]EGF was 1–2 ×
10^5 cpm/ng. [35S]Methionine was purchased from PerkinElmer Life
Sciences. Pfu polymerase and QuickChange site-directed mutagenesis
kit were from Stratagene Cloning Systems (La Jolla, CA). Tetracycline,
puromycin, FP2, FP3, and PD168393 were from Calbiochem-Novabio-
chem from Invitrogen, respectively. streptavidin-coated magnetic
microbeads, and m2 subunit was kindly provided by Dr. M. S. Robinson
(Cambridge University, Cambridge, UK). Polyclonal antibodies Ab2931 to
EGFR were provided by Dr. L. Beguinot (DIBIT Raffaie, Milan, Italy). Mon-
clonal antibodies to EGFR (Ab528), α subunit of AP-2 (AP.6), and
clathrin heavy chain (X.22) were obtained from American Type Culture
Collection (Manassas, VA); monoclonal AC1 antibody to α-adaptin was
from Affinity Bioreagents, Inc. (Golden, CO). Monoclonal antibodies
RC29 conjugated with horseradish peroxidase and 4G10 specific to
phosphotyrosine were purchased from BD Biosciences and Upstate
Biotechnology, Inc. (Lake Placid, NY), respectively. Rabbit polyclonal
antibody Ab32 specific to β-adaptins was described previously (19).
SU6566 was kindly provided by SUGEN, Inc. Monoclonal antibody
32-CFP fusion proteins, respectively. All point mutations in
β2-YFP were made using QuickChange mutagenesis kit according to
the manufacturer’s protocol (Stratagene). To generate YFP-tagged μ2 sub-
unit of AP-2, μ2 internally tagged with HA epitope in pcDNA3.1 (15)
was used as a template. Full-length YFP was amplified by PCR and
cloned into an Xhol restriction site between a HA tag and the carboxy-
terminal sequence of μ2 (corresponding to residues 237–435). All con-
 structs and point mutations were verified by dideoxynucleotide
sequencing.

Cell Culture and Transfections—HeLa cells were grown in Dulbecco’s
modified Eagle’s medium (DMEM) containing 10% fetal bovine serum,
antibiotics, and glutamine. Porcine aortic endothelial (PAE) cells ex-
grown in F12 medium containing 10% fetal bovine
serum, antibiotics, and glutamine. Human epidermal carcinoma A-431
PAE cells were grown in Dulbecco’s modified Eagle
medium at 37
° C for 1 h followed by three rinses with cold
DMEM to neutralize the acid. This acid wash procedure does not affect
the binding properties of EGFR (23). The cells were then incubated with
4.25 nM 125I-EGF at 4
° C for 1 h followed by three rinses with cold
DMEM to remove unbound 125I-EGF. The cells were lysed in 1 m
NaOH at 1 h at room temperature to determine cell-bound radioactivity.
Nonspecific binding was measured for each time point in the presence
of 100-fold molar excess of unlabeled EGF and was not more than
5–10% of the total counts.

**Results**

**β2 Subunit of AP-2 Is Tyrosine-phosphorylated**—In search of
tyrosine-phosphorylated proteins that interact with the region
of the EGFR important for receptor internalization (residues
945–1022), we performed affinity-binding experiments with the corresponding recombinant protein and lysates of EGFR-
stimulated HeLa cells. Immunoblotting with phosphotyrosine
antibodies detected only one protein band with an apparent
molecular mass of ~100 kDa that was specifically bound to
the 945–1022 EGFR fragment. Mass spectrometry analysis identi-
fied β2 subunit of AP-2 in this Coomassie-stained band. These
experiments prompted us to investigate whether β2-adaptin is
indeed tyrosine-phosphorylated in cells stimulated with EGF.
To demonstrate directly β2 tyrosine phosphorylation, AP-2

**Fluorescence Imaging**—HeLa cells transiently expressing β2-YFP,
β2-CFP, and/or μ2-YFP protein for 2–3 days or PAE cells lining
expressing wild-type or mutant EGFR were grown on glass coverslips. The cells were imaged live using CFP and YFP filter channels as described (21). In
immunofluorescence experiments, the cells were washed with CMF-
PBS and fixed with freshly prepared 4% paraformaldehyde (Electron
Microscopy Sciences, Ft. Washington, PA) for 12 min at room temper-
ature. The cells were mildly permeabilized using a 3-min incubation in
CMF-PBS containing 0.1% Triton X-100 and 0.5% BSA at room tem-
perature, further incubated in CMF-PBS containing 0.5% BSA at room
temperature for 1 h with monoclonal AP-6 antibody to α-adaptin, X.22
polyclonal antibody to clathrin heavy chain, or Ab528 antibody to EGFR,
and then incubated for 30 min with the secondary donkey anti-mouse
IgG labeled with Cy3 (The Jackson Laboratories, West Grove, PA). Both
primary and secondary antibody solutions were precluded by cunf-
trification at 100,000 × g for 10 min. After staining the coverslips were
mounted in Fluromount-G (Southern Biotech Inc., Birmingham, AL)
containing 1 mg/ml para-phenylenediamine. In experiments with PAE
clones, the cells were incubated with 1 μg/ml 4,6-diamidino-2-phenylin-
dole (DAPI) for 5 min at room temperature to label cell nuclei. Three-
dimensional images of cells were obtained as described (22) using an
epifluorescence imaging work station equipped with a × 100 oil immer-
sion objective lens, cooled CCD, x-y step motor, dual filter wheels, and
a xenon 150-watt light source, all controlled by SlideBook 3.0 software
(Intelligent Imaging Innovation, Denver, CO). Typically, 20–30 serial
two-dimensional images were recorded at 200-nm intervals. A Z-stack
of images obtained was deconvoluted using a modification of the con-
strained iteration method. Final arrangement of all images was per-
formed using Adobe Photoshop.

**Immunoprecipitation of [35S]Methionine-labeled EGFR**—PAE cells line
pressing wild-type or mutant EGFR were grown in 35-mm dishes and
metabolically labeled overnight with [35S]methionine (50 μCi per dish)
in methionine-free DMEM containing 1% dialyzed fetal bovine serum.
The cells were washed twice with binding medium (DMEM + 0.1%
BSA) and then incubated for 1 h in binding medium at 37
° C. The cells
were rinsed once more with binding medium and further incubated
with or without 34 nm EGF in binding medium for the times indicated.
At the end of the incubation, the cells were briefly rinsed with ice-
cold CMF-PBS, and the plates were frozen at –80
° C. After all incubations
were completed, all cells were thawed and solubilized in a Triton X-100
lysis buffer supplemented with 1% deoxycholate. EGFR was immuno-
precipitated with the antibody Ab293, and the immunoprecipitates
were resolved on 7.5% SDS-PAGE. The gel was dried and exposed to
x-ray film, and the amount of [35S]-labeled EGFR in immunoprecipitates
was quantitated by densitometry.

**EGFR Down-regulation**—To monitor EGFR down-regulation, cells
grown in 12-well dishes were incubated with EGF (34 nm) in binding
medium at 37
° C. After the indicated times, the cells were rinsed with
ice-cold DMEM to remove unbound EGF and then incubated with
ice-cold 0.2 nm sodium acetate buffer, pH 4.5, containing 0.5 mM NaCl
for 2.5 min to remove surface-bound EGF followed by two rinses with cold
DMEM to neutralize the acid. This acid wash procedure does not affect
the binding properties of EGFR (23). The cells were then incubated with
4.25 nm 125I-EGF at 4
° C for 1 h followed by three rinses with cold
DMEM to remove unbound 125I-EGF. The cells were lysed in 1 m
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**RESULTS**

Tyrosine phosphorylation of AP-2

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A-431 cells were stimulated with EGF (34 nM) for 10 min at 37 °C and lysed. AP-2 complexes were immunoprecipitated (IP) with antibody AP.6. Normal mouse IgG2b was used as control for the specificity of immunoprecipitation. AP-2 immunoprecipitates were resolved on 7.5% SDS-PAGE with or without 6 M urea and analyzed by Western blotting with antibody RC20-HRP to phosphotyrosine (pTyr), Ab32 to AP-2-adaptn (β2), and AC-1 to α-adaptn (α). B, A-431 and HeLa cells were treated with EGF (17 nM) for indicated times. AP-2 complexes were immunoprecipitated as in A. C, HeLa cells were incubated with 17 nM EGF at 4 °C for 45 min or at 37 °C for 5 min and then lysed. AP-2 complexes were immunoprecipitated as in A. Immunoprecipitates in B and C were analyzed as described in A.

was immunoprecipitated from HeLa cells, and the presence of tyrosine-phosphorylated proteins in immunoprecipitates was tested by Western blotting (Fig. 1A). The tyrosine phosphorylation of a 100-kDa band that precisely overlapped with the α-adaptin band was detected in cells treated with EGF. Because αC isoform of α subunit of AP-2 runs on SDS gels similar to β2, the two subunits were separated using urea-SDS gels (24). Under these conditions β2 migrates significantly slower than αA and αC subunits (the latter is expressed at very low levels in HeLa cells). The tyrosine phosphorylation of a slow-moving β2 subunit (~118 kDa) was detected in urea gels, although the phosphotyrosine signal associated with β2 was weaker than in SDS gels (Fig. 1A). These results suggested that β2 adaptin is phosphorylated in an EGF-dependent manner.

The intensities of the phosphotyrosine antibody signal associated with β2 on Western blots were very low in HeLa cells expressing moderately low levels of EGFRs. The signal was detectable several minutes after continuous EGF treatment at 37 °C and then decreased with time. In A-431 cells, expressing high levels of EGFRs, the tyrosine phosphorylation signal was much stronger and persistent during at least 30 min of EGF stimulation (Fig. 1B). β2 was not immunoprecipitated by several phosphotyrosine antibodies from cell lysates, presumably because the tyrosine-phosphorylated sequence of β2 was not accessible to antibodies and/or the amount of phosphorylated β2 molecules was very small compared with the total amount of tyrosine-phosphorylated proteins in lysates of cells treated with EGF.

β2 was only moderately phosphorylated when cells were stimulated with EGF at 4 °C (Fig. 1C), conditions allowing EGF activation, recruitment to coated pits but restricting endocytosis (22). For the maximum high level of phosphorylation, the incubation of cells with EGF at 37 °C was required (Fig. 1C).

Inhibitors of EGFR Kinase Block β2 Phosphorylation—EGFR activation leads to activation of Src kinases that are responsible for tyrosine phosphorylation of clathrin heavy chain (25) and dynamin (26), the major functional components of coated pits. To determine whether EGFR kinase directly phosphorylates β2, or β2 phosphorylation is mediated through the activation of Src family kinases, the effect of EGFR kinase inhibitor (27) reduced β2 phosphorylation was compared with that of Src inhibitors. PD168393 completely blocked tyrosine phosphorylation of β2 in A-431 cells (Fig. 2). Src-family kinase inhibitor PD2 reduced β2 phosphorylation as compared with cells treated with the control compound, PP3. However, PP2 also partially inhibited EGFR kinase activity as evident from the decreased phosphorylation of the EGFR (also see (22)). A more specific Src family kinase inhibitor SU6656 (27) reduced β2 phosphorylation in A-431 cells by 25–30%. However, the same inhibitor did not affect β2 phosphorylation in HeLa cells (Fig. 2). These data suggested that β2 is phosphorylated either directly by the EGFR kinase or by another Src-unrelated tyrosine kinase that is activated by the EGFR. Src family kinases may contribute to β2 tyrosine phosphorylation in cells, such as A-431 cells, in which these kinases are effectively activated by EGFR.

Functional β2-YFP Fusion Protein Is Tyrosine-phosphorylated—To map tyrosine phosphorylation sites in β2 adaptin, β2 tagged with YFP was ectopically expressed in HeLa cells. β2-YFP was efficiently immunoprecipitated using antibodies to α-adaptin of AP-2, indicating that β2 fusion protein is incorporated into the AP-2 complex (Fig. 3A). Typically, 30–50 and
Tyrosine Phosphorylation of AP-2

Fig. 3. Incorporation of β2-YFP and μ2-YFP into AP-2 and tyrosine phosphorylation of β2-YFP. A, HeLa cells transfected with β2-YFP construct or mock-transfected for 2 days were lysed, and AP-2 complexes were immunoprecipitated (IP) with using AP.6. β2-YFP and endogenous β2 were detected by Western blotting of immunoprecipitates with Ab32. B, HeLa cells expressing β2-YFP for 2 days were treated with EGF (17 nM) for 5 min at 37°C and lysed, and AP-2 complexes were immunoprecipitated using antibody AP.6. Tyrosine-phosphorylation of β2-YFP and endogenous β2 was detected by Western blotting using anti-phosphotyrosine antibodies RC20-HRP (pTyr) or 4G10. C, HeLa cells transfected with HA-μ2-YFP or mock-transfected were lysed, and AP-2 was immunoprecipitated using antibody AP.6. HA-μ2-YFP was detected in cell lysates and immunoprecipitates using anti-HA antibody, whereas α-adaptins were detected using antibody AC.1.

20–30% of total cellular β2-YFP and endogenous β2, respectively, remained in supernatants after immunoprecipitation due to incomplete immunoprecipitation of AP-2 complexes and, possibly, the presence of free β2-YFP and β2 not assembled into AP-2 (data not shown). Blotting of AP-2 immunoprecipitates with two different antibodies to phosphotyrosine yielded signals from both endogenous β2 and heterologously expressed β2-YFP (Fig. 3B). These data indicate that β2-YFP incorporated into AP-2 is tyrosine-phosphorylated in an EGF-dependent manner.

In living cells β2-YFP (data not shown) and β2-CFP (Fig. 4A) were localized in small dots throughout the cell, a pattern of distribution typical of coated pits. When β2-CFP was co-expressed with the μ2 subunit of AP-2 tagged with YFP, both subunits were highly co-localized (Fig. 4A). Immunoprecipitation experiments showed that μ2-YFP was effectively incorporated into AP-2 complex (Fig. 3C), thus confirming that the localization of both β2-CFP and μ2-YFP corresponds to localization of AP-2. Furthermore, β2-YFP was well co-localized in fixed cells with endogenous clathrin heavy chain and α-adaptin (Fig. 4B), suggesting that β2-YFP is correctly targeted to coated pits. In some cells, a pool of β2-YFP was seen in the Golgi area. It is possible that overexpressed β2-YFP can incorporate into AP-1 complex located in the Golgi. Alternatively, overexpressed β2-YFP may form aggregates that concentrate in the Golgi area.

Tyrosine 6 Is the Major Phosphorylation Site in β2—To map phosphorylation sites in β2, we tested whether mutations of individual tyrosines in β2-YFP yield reduced phosphorylation of this fusion protein. Mutation of tyrosine 6 to phenylalanine decreased phosphotyrosine signal associated with β2-YFP by 50% (Fig. 5). Single mutations of all 23 other tyrosines in the trunk and hinge domains (residues 1–844) did not affect significantly tyrosine phosphorylation of β2-YFP. When Y6F mutation was combined with several other mutations (up to six Tyr-to-Phe mutations), no further decrease in phosphotyrosine signal was observed. Truncated version of β2-YFP-Y6F, in which the ear domain was removed (1–844), was phosphorylated to the same extent as the full-length Y6F mutant (Fig. 5). These data suggest that Tyr-6 is important for phosphorylation of β2 in cells stimulated with EGF, but there are other phosphorylation sites, which were not identified by mutagenesis. These additional sites are probably located within the amino-terminal trunk domain of β2. In support of this notion, tyrosine phosphorylation was detected in the trunk but not ear/hinge fragments of endogenous β2 protein that was proteolytically cleaved with elastase or trypsin (data not shown).

EGFR Phosphorylates β2 through Its Interaction with μ2 and Possibly Direct Interaction with β2.—We have shown previously that EGFR directly binds μ2 subunit of AP-2 and that Tyr-974-containing motif of the receptor is responsible for this interaction (14, 15, 28). Does this interaction with AP-2 mediate β2 phosphorylation? To test this possibility, EGFR mutants that were stably expressed in PAE cells were utilized (20). Stimulation of PAE cells expressing EGFR-Y974A mutant produced impaired tyrosine phosphorylation of β2 as compared with wild-type EGFR expressing cells (Fig. 6A). This result suggested that the interaction of the 974YRAL motif of EGFR with AP-2 is partially responsible for β2 phosphorylation.

To confirm this conclusion directly, HeLa cells that inducibly express mutant μ2 incapable of interaction with the EGFR were used (15). In these cells the induction of the expression of HA-tagged μ2 D176A/W421A mutant upon removal of tetracycline results in efficient replacement of endogenous μ2 from AP-2 complexes with the mutant μ2 (Fig. 6B), thus preventing the interaction of EGFR with AP-2 through μ2. Expression of mutant μ2 (minus tetracycline) significantly reduced EGF-dependent tyrosine phosphorylation of β2 (Fig. 6B). These data suggested that the interaction of EGFR with μ2 contributes to β2 phosphorylation. Together, the experiments with mutants of EGFR and μ2 indicate that the direct interaction of 974YRAL motif with μ2 partially mediates β2 phosphorylation.

To define other mechanisms of β2 phosphorylation by the EGFR, several PAE cell lines expressing EGFR mutants in putative internalization and AP-2-binding motifs were used (20). Fig. 6A shows that the EGFR mutant, in which leucines 1010 and 1011 were replaced by alanines, weakly phosphorylated β2. These experiments suggested that Leu-1010/Leu-1011 motif is critical for EGF-dependent β2 phosphorylation in PAE cells.

Leucines 1010 and 1011 Are Important for EGFR Turnover—Because Leu-1010/Leu-1011 motif is important for β2 phosphorylation, we speculated that this motif may regulate EGFR endocytosis. Previous studies showed that Leu-1010/Leu-1011 is not critical for internalization of EGFR through a rapid saturable pathway, although we observed some variation in the internalization rates among different clones of PAE cells ex-
Images of live cells were acquired through the YFP and CFP channels. Aldehyde and stained with monoclonal antibodies to IgG conjugated with Cy3. The serial z-optical sections (thickness 0.2 μm) were acquired through the YFP and CFP channels. After prolonged exposure of cells to EGF (2 h), mutant EGFR resulted in the impaired EGF-dependent down-regulation of the cell-surface receptors (Fig. 7B). In both cell lines, receptors were well co-localized with EEA.1 protein, a marker of early and intermediate endosomes during 2 h of continuous endocytosis (data not shown). The slow turnover of mutant EGFR resulted in the impaired EGF-dependent down-regulation of the cell-surface receptors (Fig. 7C). Altogether, the data in Fig. 7 suggested that Leu-1010/Leu-1011 motif is involved in the regulation of the endosomal sorting of EGFR to the degradative pathway.

The slow degradation of mutant EGFR was also observed by fluorescence microscopy. After a 15-min stimulation of the cells with EGF, a similar pattern of endosomal localization of wild-type and L1010A/L1011A receptors was observed (Fig. 7B). After prolonged exposure of cells to EGF (2 h), mutant EGFR remained accumulated in the vesicular compartments, whereas a significant decrease in EGFR immunoreactivity was observed in cells expressing wild-type EGFR (Fig. 7B). In both cell lines, receptors were well co-localized with EEA.1 protein, a marker of early and intermediate endosomes during 2 h of continuous endocytosis (data not shown). The slow turnover of mutant EGFR resulted in the impaired EGF-dependent down-regulation of the cell-surface receptors (Fig. 7C). Altogether, the data in Fig. 7 suggested that Leu-1010/Leu-1011 motif is involved in the regulation of the endosomal sorting of EGFR to the degradative pathway.

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Protein phosphorylation is the major regulatory mechanism of clathrin-dependent endocytosis. In particular, serine and threonine phosphorylation of clathrin coat proteins plays an important role in the organization of macromolecular complexes during clathrin-mediated endocytosis (16, 29). Tyrosine phosphorylation of coat proteins, such as clathrin heavy chain and dynamin, has been observed in cells stimulated with growth factors or hormones, although the precise role of these modifications is not fully understood (25, 26). Hence we demonstrated tyrosine phosphorylation of the β2 subunit of AP-2 complexes in cells stimulated with EGF. This is the first observation of tyrosine phosphorylation of AP complexes.

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Tyrosine Phosphorylation of AP-2

Fig. 7. Degradation and down-regulation of L1010A/L1011A EGFR mutant. A, PAE cell lines expressing wild-type EGFR (WT) or L1010A/L1011A mutant (clones LL#1 and LL#16) were metabolically labeled with [35S]methionine followed by incubation with or without EGF (34 nM) in binding medium at 37 °C for the times indicated. The cells were then lysed, and EGFRs were immunoprecipitated with Ab528. The immunoprecipitates were separated by SDS-PAGE, and the amount of [35S]-labeled EGFR in immunoprecipitates was determined by autoradiography and quantitated by densitometry. The amount of labeled EGFR is expressed as percent of this amount at time point 0. The data represent mean values from three independent experiments. B, WT and LL1 cells grown on coverslips were incubated with 34 nM EGF at 37 °C for 15 min or 2 h, fixed and stained with monoclonal antibody to EGFR (Ab528), followed by secondary donkey anti-mouse IgG conjugated with Cy3. Cellular nuclei were labeled by DAPI. The images were acquired through the Cy3 (red) and DAPI (blue) channels with the same integration time for each experimental condition. C, wild type (WT), LL1, and LL16 cells grown in 12-well plates were incubated with 34 nM EGF at 37 °C for the indicated times. The residual number of surface EGFR was determined using 125I-EGF binding assay as described under “Experimental Procedures” and expressed as percent to that number at time point 0.

Because β2 phosphorylation was completely blocked by the EGFR kinase inhibitor and only partially inhibited by the Src kinase family inhibitor (Fig. 2), it is likely that β2 is a substrate of the EGFR kinase. It cannot be, however, ruled out, that β2 is phosphorylated by an unidentified tyrosine kinase activated by the EGFR. In A-431 cells, c-Src activated by EGFR may directly phosphorylate β2 or act by maximally activating the EGFR kinase through phosphorylation of tyrosine 845 in the kinase domain of the receptor. Interestingly, AP-2 was continuously phosphorylated (Fig. 1) and associated with EGFR after internalization in A-431 cells (28). It is therefore possible that β2 was tyrosine-phosphorylated after internalization in A-431 cells and that Src is involved in endosomal phosphorylation of AP-2 in these cells. A low level of β2 phosphorylation was observed at 4 °C, conditions restricting endocytosis but allowing maximum recruitment of receptors into coated pits (22).

The extent of tyrosine phosphorylation of β2 was maximally high after cell treatment with EGF at 37 °C, suggesting that β2 phosphorylation occurs mainly during a post-recruitment step. This is consistent with the notion that the interaction with AP-2 is not necessary for the initial stages of EGFR endocytosis (15).

To examine the functional role of AP-2 tyrosine phosphorylation, we attempted to map phosphorylation sites, which would allow us to generate a dominant-interfering mutant of β2. However, we have not been able to map all phosphorylation sites. Mutation of Tyr-6 caused a 50% reduction of tyrosine phosphorylation signal, suggesting that this residue could be the major phosphorylation site. This tyrosine is located in proximity to phosphatidylinositol-binding site on the surface of AP-2 core complex and is accessible for phosphorylation (4). Extensive mutagenesis of other tyrosines in β2-YFP did not reveal other phosphorylation sites. Furthermore, tandem nanospray mass spectrometry analysis did not reveal additional tyrosine phosphorylation sites. It is possible that multiple tyrosines are phosphorylated in addition to Tyr-6, and the mutations of these tyrosines are compensated by the phosphorylation of other tyrosines. It is also possible that phosphotyrosine antibodies cross-react with phosphoserine- or phosphothreonine-containing sequences in β2. In fact, mass spectrometry revealed phosphorylation of Ser-563 and Thr-647 of β2 (data not shown). Mutations of these residues slightly reduced the phosphotyrosine antibody signal associated with β2 (data not shown). However, this effect can be attributed to the conformational changes caused by mutations. Finally, we cannot formally rule out the possibility that the residual phosphotyrosine signal in Y6F-β2-YFP is due to tyrosine phosphorylation of the YFP moiety in the β2 fusion protein. The interaction of EGFR with AP-2 has been demonstrated by co-immunoprecipitation and in vitro assays with purified proteins (30–32). The observation of EGFR-dependent AP-2 tyrosine phosphorylation provides yet another evidence for the interaction of EGFR and AP-2 in intact cells. However, the role of this interaction remains
unclear. It has been shown by several experimental approaches that the elimination of EGFR-μ2 interaction does not significantly affect EGFR internalization (14, 15). In this study we show that leucines 1010 and 1011 in EGFR are important for β2 phosphorylation. This observation implies that the receptor LL motif may engage into interactions with AP-2. Such interactions must be, however, weak and unstable in detergent extracts, because they could not be detected by co-immunoprecipitation. It is also possible that the mutation of leucines 1010/1011 affects the conformation of the receptor carboxyl terminus, thus indirectly influencing the ability of EGFR to phosphorylate AP-2. As in the case of mutants of Tyr-974-containing motif, Leu-1010/Leu-1011 mutants of EGFR displayed high internalization rates (20). Therefore, the Leu-1010/Leu-1011 motif is unlikely to have a significant role in clathrin-dependent internalization of EGFR.

Surprisingly, L1010A/L1011A mutant of EGFR expressed in PAE cells displayed a substantially reduced rate of the turnover (Fig. 7). The data are consistent with the model by which this LL motif is involved in the regulation of lysosomal targeting of unoccupied and occupied EGFR. Such function may be related to a proposed interaction of this motif with AP-2 (Fig. 6). Previous studies (28, 33) suggested that AP-2 can be detected in endosomes and lysosomes and that EGFR/AP-2 interaction may persist after receptor internalization. Alternatively, L1010A/L1011A motif may function in intracellular sorting independently on its role in AP-2 phosphorylation. Interestingly, Leu-679/Leu-680 motif in the juxtamembrane domain of EGFR was implicated into lysosomal targeting of the EGFR (34). However, mutation of the juxtamembrane LL motif affected folding of the EGFR molecule, suggesting that these residues could have an indirect role in the lysosomal targeting of the receptor (35).

Recently, the role of EGFR ubiquitination in endosomal sorting has been demonstrated using several experimental approaches (36, 37). In our experiments, the level of ubiquitination of wild-type and L1010A/L1011A mutant EGFR was not significantly different (data not shown), suggesting that the function of LL motif could be downstream of the ubiquitination step. It is possible that prolonged association of EGFRs with AP-2 after internalization interferes with recycling of EGFR from early endosomes, thus facilitating binding of EGFR to endosomal sorting complexes containing ubiquitin-binding proteins. Future studies should elucidate how di-leucine sequences and ubiquitin moieties are recognized by the endosomal machinery, and whether these two types of signals represent sequential or redundant steps of the EGFR endosomal sorting.

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