Ligand-induced Internalization and Increased Cell Calcium Are Mediated via Distinct Structural Elements in the Carboxyl Terminus of the Epidermal Growth Factor Receptor*

(Received for publication, July 23, 1991)

Chia-Ping Chang††, Joseph P. Y. Kao†, Cheri S. Lazar‡, Brenda J. Walsh**, Alan Wells‡ ‡, H. Steven Wiley**, Gordon N. Gilli, and Michael G. Rosenfeld‡‡††

From the †Eukaryotic Regulatory Biology Program, Departments of ‡Medicine and §§Pathology, and ‡‡Howard Hughes Medical Institute, University of California at San Diego, La Jolla, California 92093, the †Department of Physiology, University of Maryland at Baltimore, Baltimore, Maryland 21201, and the §§Department of Pathology, University of Utah, Salt Lake City, Utah 84132

Signals that can mediate ligand-induced receptor internalization and calcium regulation are present in a 48-amino acid “calcium-internalization” domain in the C′ terminus of the epidermal growth factor (EGF) receptor. The basis of calcium and internalization regulation signalled by this 48-amino acid sequence was analyzed using deletion and substitution mutant receptors. Cells expressing truncated receptors containing either the NH₂- or COOH-terminal portion of the 48-residue domain displayed high affinity EGF-dependent endocytosis and receptor down-regulation. These endocytosis-competent EGF receptor mutants that lacked any autophosphorylation site were unable to increase the concentration of intracellular calcium. To investigate the role of self-phosphorylation in EGF-induced calcium mobilization, phenylalanine was substituted for the single autophosphorylated tyrosine residue in this region of an internalization-competent truncated receptor. The receptor-mediated calcium response was abolished, while ligand-dependent receptor internalization was unimpaired. These results demonstrate that EGF-dependent receptor endocytosis and calcium mobilization are separate events. Tyrosine self-phosphorylation is required for increased [Ca²⁺], while structural features distinct from autophosphorylation are required for receptor internalization.

Ligand-dependent activation of the intrinsic protein tyrosine kinase activity of growth factor receptors is essential for transducing signals required for the biological responses of the cell. Kinase-inactive (Kin−) EGF receptors fail to signal either early or late responses to the growth factor (1, 2) and also fail to undergo ligand-induced internalization and down-regulation (3–5). Analysis of a series of EGF receptors containing progressive C′-terminal deletions indicated that the distal boundary of the kinase domain is located at residue 957 (5), corresponding to an exon/intron junction in the gene (6). The 229 amino acids C′ terminus distal to residue 957 are required for EGF-induced increases in cytosolic Ca²⁺ concentrations ([Ca²⁺]), receptor endocytosis, and down-regulation (5). This region contains five tyrosine autophosphorylation sites (7–9). Based on differential functions of receptors truncated at residues 973 and 1022, this 48-amino acid region in the regulatory C′ terminus was sufficient to confer both calcium regulation and endocytosis of receptors. The region from 973 to 1022 was therefore referred to as the calcium-internalization (CaIn) domain (5).

In this article we investigate whether the structural requirements for EGF-induced receptor internalization and calcium responses are conferred by separate signals. Permanent cell lines expressing EGF receptors with deletions or specific mutations within the CaIn domain were analyzed for EGF-induced increases in [Ca²⁺], and for ligand-induced internalization and down-regulation. Sequences critical for receptor internalization are distinct from a requirement of tyrosine self-phosphorylation for EGF-induced increases in [Ca²⁺]. These results indicate that ligand-induced increases in [Ca²⁺], and endocytosis are mediated by distinct events which depend on different sequence motifs, each of which is located in the regulatory C′ terminus of the EGF receptor.

MATERIALS AND METHODS

Construction and Expression of Mutant Receptors—The expression plasmids containing the hEGF receptor cDNA, the kinase-inactive mutant (5), and C′-terminal truncated EGF receptors (c′991, c′973, and c′957) have been described (1, 5). A truncation mutant (c′1000) was obtained by removing the coding sequence for the last 186 amino acids of the EGF receptor. To generate the fusion plasmid p578/993–1022, a SalI site was created at residues 959 and 992 by site-directed mutagenesis according to the method of Kunkel (10). The sequences between the two SalI sites were deleted, and residues Val and Asp were introduced at the junction. In the plasmid c′1000F, the Tyr at residue 992 was substituted by Phe. All the mutant sequences were confirmed by the dideoxy chain termination method. Transfection and selection of monoclonal mouse B82 L cells expressing the various EGF receptor mutants were carried out as previously described (1). Colonies were screened using 125I-EGF binding (11).

Receptor Down-regulation—Cells expressing normal or mutant EGF receptors were treated with 50 nM EGF for various times at 37 °C. The extent of receptor down-regulation was determined by 125I-EGF binding after acid stripping (12). Nonspecific binding, estimated in the presence of a 200-fold molar excess of unlabeled EGF, was less than 5% and was subtracted. Measurements were made in at least two independent clonal B82 cell lines expressing each receptor.
Internalization of EGF Receptors.—The specific internalization rate (ke) in B82 cells expressing normal or mutant receptor was measured as described by Lund et al. (13). The affinity and capacity of the endocytic system for ligand-bound receptors was evaluated by plotting ke against the net internalization velocity measured at varying ligand concentrations. This generates a saturation of internalization (Satin) plot (13).

Measurement of Intracellular Calcium—Cells were grown on coverslips in complete culture medium, then shifted to serum-free medium containing 0.5 mg/ml of bovine serum albumin at least 15 h before experiments. Cells were loaded with the fluorescent Ca²⁺ indicator fura-2 via the acetoxymethyl ester for 40-60 min at room temperature; quantification was performed as described (1, 14). Cells were exposed to 0.5% fetal bovine serum as a positive control. To document cell viability, 5 μM ionomycin was added to demonstrate ability to increase [Ca²⁺].

Immunodetection of Autophosphorylated EGF Receptors—Cells were incubated without or with 50 nM EGF for 2 min at 37 °C. After washing, cells were solubilized and the receptors were immunoprecipitated with 1 μM monoclonal mouse anti-EGF receptor 528 antibody (16). The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (9). To detect receptor autophosphorylation, blots were incubated with 1:5 PY20 monoclonal antiphosphotyrosine antibody at 1:1000 dilution (4). Membranes were then washed followed by autoradiography. EGF receptors were detected on the same blots by incubation with a rabbit anti-hEGF receptor antibody directed against the N′-terminal 13 amino acids of the hEGF receptor diluted 1:1000 in the same blocking buffer. The washed membranes were developed by the alkaline phosphatase-conjugate detection system (Promega).

RESULTS

Characterization of the Internalization Domain of the EGF Receptor—Previous analysis of mutant EGF receptors with truncated C′ termini indicated that receptor internalization could be restored by a region spanning residues 973-1022 (5), implying that these sequences are a critical component of the internalization signal. Examination of the primary amino acid sequence of this domain suggests distinct regions. The proximal portion (residue 973-991), rich in acidic residues, is highly negatively charged, while the distal portion (residue 992-1022) is a Ser/Thr-rich region. It has been established that there is a single autophosphorylation site at Tyr-992 (9) and there are sites of serine phosphorylation in the Ser/Thr-rich region (16). To evaluate the contribution of each subregion to the process of ligand-induced internalization and down-regulation, two mutant EGF receptors were constructed. One was truncated at residue 991 (c′991); the other was truncated at residue 958 with residues 993-1022 fused at the C′ terminus (f958/993-1022). Plasmids expressing either transcription unit were transfected into mouse B82 cells that do not express endogenous EGF receptors.

Receptors—Cells expressing the indicated EGF receptors using 125I-EGF concentrations ranging from 0.17 to 18 nM. Data are presented as Satin plots, which are analogous to Scatchard plots, for holo and mutant EGF receptors. As shown in Fig. 1, the Satin plot of the holo receptor is biphasic, indicating entry through both high affinity and constitutive endocytic pathways (13, 18). In contrast, kinase-active receptors truncated to residue 973 show a slope of near 0, indicating that these receptors lack sequences required for high affinity internalization. Similar results were observed for c′957, kinase-active EGF receptors (5). Addition of the sequences of the distal part of the CaIn domain (residues 993-1022) to the kinase core, f958/993-1022, restored high affinity endocytosis (Fig. 1). Similarly, EGF receptors containing the proximal portion of the CaIn domain (c′991) also exhibited biphasic Satin plots (Fig. 1), indicating that sequences located between residues 973 and 991 are also capable of conferring high affinity endocytosis. Both the distal and proximal halves of the CaIn domain thus contain sequences that contribute to occupancy induced high affinity internalization of holo EGF receptors.

Uncoupling of Ligand-Induced Calcium Response from Receptor Internalization—The identification of two internalization elements in the C′ terminus of the EGF receptor led to the investigation of the mechanisms of the uncoupling of high affinity internalization from high affinity calcium mobilization. This was achieved by measuring calcium mobilization within cells transfected with the truncated receptors using the fluoro indicator fura-2 via the acetoxymethyl ester for 40-60 min at room temperature; quantification was performed as described (1, 14). Cells were exposed to 0.5% fetal bovine serum as a positive control. To document cell viability, 5 μM ionomycin was added to demonstrate ability to increase [Ca²⁺].

FIG. 1. High affinity internalization of mutant EGF receptors that contain different C′ termini. The values of ke and internalization velocity were determined in monoclonal B82 cells expressing the indicated EGF receptors using 125I-EGF concentrations ranging from 0.17 to 18 nM. Data are presented as Satin plots (13). The curve through the data was fit to a two-component internalization model (high affinity and constitutive internalization) using the simplex algorithm (18). Similar results were obtained in four independent experiments using different permanent transfections.
Fig. 2. Down-regulation of mutant EGF receptors. The kinetics of surface receptor loss were measured at the indicated times following addition of 50 nM EGF at 37 °C as described under “Materials and Methods.” Results are the mean for three to seven determinations which differed by less than 10%.

![Down-regulation of mutant EGF receptors](image)

Fig. 3. EGF-induced changes in \([\text{Ca}^{2+}]\), in B82 cells expressing the indicated EGF receptors. Cells grown on coverslips were loaded with fura-2 and monitored for \([\text{Ca}^{2+}]\) by digital video fluorescence microscopy. Arrows indicate the addition of 50 nM EGF. Each dot represents the \([\text{Ca}^{2+}]\), of a single cell selected at random across a microscopic field. The solid line superimposed on each dot plot represents the mean of the 24 cells examined in each panel.

![EGF-induced changes in Ca^2+](image)

us to further evaluate their role in the EGF-dependent calcium regulation conferred by the CaIn domain. Because the EGF-induced calcium response is rapid and transient, accurate continuous measurements of intracellular calcium concentration are necessary. Ratio imaging of individual cells was performed to determine changes in calcium levels over time. Images were acquired at regular intervals, allowing for the visualization of calcium transients.

---

**Table I**

**EGF-induced changes in \([\text{Ca}^{2+}]\).**

| Receptor       | \([\text{Ca}^{2+}]\), response as % given by cells with holoreceptor |
|----------------|-----------------------------------------------------------------------|
| Holo           | 100                                                                   |
| M^721 1022 (Kin+) | 13 ± 11                                                                |
| c'1000         | 74 ± 14                                                                |
| c'1000 P^F592  | 11 ± 5                                                                 |
| f958/993-1022  | 9 ± 7                                                                  |
| c'991          | 4 ± 13                                                                 |
| c'957          | 7 ± 3                                                                  |

---

![Analysis of self-phosphorylation of mutant EGF receptors in vivo](image)

EGF-stimulated \([\text{Ca}^{2+}]\). Requires EGF Receptor Autophosphorylation—The dramatic consequence of deletion of amino acid residues 1000–991 to extinguish EGF-stimulated increases in \([\text{Ca}^{2+}]\), indicated this region contained a potent signal for regulating \([\text{Ca}^{2+}]\). Because 8 out of 9 amino acids in this region were included in f958/993-1022 EGF receptors which gave no calcium response to EGF, the amino acid at 992 (Tyr) was deduced to be the essential signal. Because Tyr-992 is the most N-terminal tyrosine self-phosphorylation site in the EGF receptor (9), we tested the hypothesis that receptor self-phosphorylation was necessary for increased \([\text{Ca}^{2+}]\). A mutant EGF receptor truncated at residue 1000 and containing a Phe substitution at 992 (c'1000 F^P592) lacked detectable tyrosine self-phosphorylation. As shown in Fig. 4, cells expressing holo and c'1000 EGF receptors exhibited EGF-induced self-phosphorylation in vivo, whereas investigated by treating cells with the membrane permeant compound BAPTA/AM, which is hydrolyzed to the active \(\text{Ca}^{2+}\) chelator BAPTA upon entry into cells (20,21). Although intracellular \(\text{Ca}^{2+}\) buffering by BAPTA was effective in blocking \(\text{Ca}^{2+}\) transients, down-regulation of holoreceptors was not affected (data not shown), providing further support for the conclusion that ligand-induced receptor internalization is independent of increased \([\text{Ca}^{2+}]\).
EGF Receptor Internalization and Calcium Response

c'1000 F° and f958/993-1022 EGF receptors did not. The c'991 EGF receptors have previously been documented to lack any detectable self-phosphorylation in vivo (9). The c'1000 F° EGF receptor was internalized and down-regulated to the same extent as c'1000 receptor (Figs. 1 and 2), but it was not competent to increase [Ca²⁺]. (Fig. 3 and Table 1). These data indicate that self-phosphorylation is not required for EGF receptor internalization but is essential for coupling receptor activation to mechanisms that increase [Ca²⁺].

DISCUSSION

Endocytosis of low density lipoprotein, transferrin, and cation-independent mannose 6-phosphate receptors is independent of ligand binding but is determined by specific cytoplasmic motifs that have been proposed to adopt a tight turn structure (22–25). This class of receptors is constitutively clustered in coated pits and continuously rapidly internalized. In contrast, a second class of receptors that serve to activate signal transduction pathways as a consequence of ligand binding undergo ligand-induced conformational changes which alter their intrinsic enzymatic activity, cell surface distribution, internalization, and trafficking in cells. For example, occupancy induced internalization of EGF and insulin receptors requires both intrinsic protein tyrosine kinase activity and specific sequence motifs (6, 18, 26, 27). Deletional analysis of the EGF receptor revealed that the sequences which are required for occupancy induced internalization via the high affinity saturable pathway are located in the C' terminus distal to the core kinase domain (5). Analysis of progressive C'-terminal truncations revealed that deletion of residues 1022–973 abolished ligand-induced internalization, down-regulation, and EGF-induced increases in [Ca²⁺]. In this article we present evidence that this 48-amino acid segment contains two distinct regions that independently specify high affinity internalization and down-regulation. Neither of these two regions, located between residues 973–991 and residues 993–1022, contain sites of tyrosine self-phosphorylation. The 993–1022 sequence appears more potent in the process of receptor down-regulation than the 973–991 sequence. The function of both regions require the intrinsic protein tyrosine kinase activity of the EGF receptor because neither sequence functions when receptor kinase activity is lost through an M' substitution. Although receptor self-phosphorylation may facilitate exposure of these sequences in the context of the holoreceptor, it is not required for their function.

The ability of EGF receptors to effect increases in [Ca²⁺], requires a site of tyrosine self-phosphorylation. Tyr-992, the most proximal of the five self-phosphorylation sites in the C' terminus, is shown to be essential for this function in the context of C'–truncated EGF receptors; and it is likely that other self-phosphorylation sites may serve this function in EGF holoreceptors (28). The self-phosphorylated C'–terminus of the EGF receptor is the binding site for phospholipase C-τ (28), an association that occurs via the SH2 domains of phospholipase C-τ (29). Phospholipase C-τ which is activated by holo EGF receptors in vivo (30), increases inositol trisphosphate, a central mediator of calcium mobilization (31). The necessity of a single site of tyrosine self-phosphorylation in the EGF receptor truncated to residue 1000 for increased [Ca²⁺] fits the postulate that interaction with phospholipase C-τ is essential to increase [Ca²⁺]. The regulatory C'–terminus of the EGF receptor contains multiple sites of tyrosine self-phosphorylation which may couple activated EGF receptors to phospholipase C-τ and increase [Ca²⁺]; Tyr-992 is demonstrated to be one such site.

Increased [Ca²⁺], is a distinct process which is not required for ligand-induced high affinity internalization and receptor down-regulation, nor for mediating growth responses. Although the sequence determinants for calcium regulation are distinct from those for occupancy induced endocytosis, both require the intrinsic protein tyrosine kinase activity of the EGF receptor. It will now be of particular interest to define precisely the sequences that signal receptor internalization, and to define the basic machinery with which these sequences interact.

Acknowledgments—We wish to thank Dr. Roger Y. Tsien for use of his imaging setup and for helpful comments on the manuscript, and Dr. Deborah Cadena for providing the EGF receptor peptide antibody.

REFERENCES

1. Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., and Rosenfeld, M. G. (1987) Nature 328, 820–823
2. Honegger, A. M., Szappary, D., Schmitz, A., Lyali, R., Van Obbergen, E., Ullrich, A. and Schlessinger, J. (1987) Mol. Cell. Biol. 7, 4568–4571
3. Honegger, A. M., Doll, T. J., Felder, S., Van Obbergen, E., Bellot, F., Szappary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1987) Cell 51, 199–209
4. Glenney, J. R., Jr., Chen, W. S., Lazar, C. S., Walton, G. M., Zokas, I. M., Rosenfeld, M. G., and Gill, G. N. (1988) Cell 52, 675–684
5. Chen, W. S., Lazar, C. S., Lund, K. A., Walsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1989) Cell 59, 33–43
6. Foley, J., Whittle, N., Bennett, P., Kirschning, D., Ullrich, A. and Waterfield, M. (1991) Oncogene Res. 1, 39–56
7. Downward, J., Parker, P., and Waterfield, M. D. (1984) Nature 311, 483–485
8. Huaan, J., Hott, N., and Waterfield, M. D. (1986) Biochem. J. 262, 659–663
9. Walton, G. M., Chen, W. S., Rosenfeld, M. G., and Gill, G. N. (1990) J. Biol. Chem. 265, 1750–1754
10. Kinkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
11. Lund, K. A., Lazer, C. S., Chen, W. S., Walsh, B. J., Walsh, J. B., Herbst, J. J., Walton, G. M., Rosenfeld, M. G., and Gill, W. H. (1990) J. Biol. Chem. 265, 20517–20523
12. Higler, H. T., Maxfield, F. R., Willingham, M. C., and Pastan, I. (1980) J. Biol. Chem. 255, 1293–1294
13. Lund, K. A., Ogysko, L. S., Starbuck, C., Walsh, B. J., and Higler, H. T. (1990) J. Biol. Chem. 265, 15713–15723
14. Ting, Y., and Hanahan, D. (1989) Cell Calcium 11, 93–109
15. Kawamata, T., Sato, J., De, A., Pollock, J., Sato, G. H., and Mendesdohn, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 80, 1337–1341
16. Heissmann, G. J., and Gill, G. N. (1988) J. Biol. Chem. 263, 13152–13158
17. Lin, C. R., Chen, W. S., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M., and Rosenfeld, M. G. (1986) Cell 44, 839–848
18. Wiley, S. H., Herbst, J. J., Walsh, B. J., Lauffenburger, D. A., Rosenfeld, M. G., and Gill, G. N. (1991) J. Biol. Chem. 266, 11083–11084
19. Moolenaar, W. H., Tootoonian, L. G. J., and de Laat, S. W. (1994) J. Biol. Chem. 269, 8066–8069
20. Tsien, R. Y. (1986) Biochemistry 19, 2396–2404
21. Tsien, R. Y. (1981) Nature 290, 527–533
22. Chen, W. S., Goldenstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 3118–3125
23. Jing, S., Spencer, T., Miller, K., Hopkins, C., and Trowsdale, J. S. (1990) J. Cell Biol. 110, 283–294
24. Collawn, J. F., Stangl, M., Kuhn, L. A., Eiseikawa, V., Jing, S., Trowsdale, J. A., and Trowsdale, J. A. (1990) Cell 63, 1091–1072
25. Russell, D. S., Gherri, R., Johnson, E. L., Chou, C.-K., and Rosen, M. O. (1987) J. Biol. Chem. 262, 11833–11840
26. Thiess, K. S., Webster, L. A., and McLaughlin, K. D. A. (1990) J. Biol. Chem. 265, 10132–10137
27. Marginoti, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Dawson, T., and Schlessinger, J. (1990) EMBO J. 9, 4373–4380
28. Akerman, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F., and Fawson, T. (1990) Science 290, 975–982
29. Honegger, A. M., Nakahata, N., Lavenberg, T. W., Dinguispe, J., Herman, B., Ehr, S. H., and Harden, T. K. (1987) J. Biol. Chem. 262, 2931–2936
30. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205

C-P. Chang, unpublished data.