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BIOSYNTHESIS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR: POST-TRANSLATIONAL GLYCOSYLATION-INDEPENDENT ACQUISITION OF TYROSINE KINASE AUTOPHOSPHORYLATION ACTIVITY*

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We previously showed that the epidermal growth factor (EGF) receptor in human A431 epidermoid carcinoma cells undergoes a slow post-translational modification whereby it acquires ($t_{1/2}$ = 30-40 min) EGF binding capacity (Slieker, L.J., et. al. (1986) J. Biol. Chem., 261, 15233-15241). This activation occurs in the endoplasmic reticulum and requires core N-linked glycosylation. By employing both anti-EGF receptor and anti-phosphotyrosine antibodies to immunoprecipitate receptor pulse-labeled with [^{35}S]methionine, we demonstrate here that the EGF receptor also acquires tyrosine kinase autophosphorylation activity post-translationally ($t_{1/2}$ = 10-15 min). The acquisition of tyrosine kinase activity is independent of the acquisition of EGF binding capacity, since it precedes the latter process and does not require N-linked glycosylation.

The binding of EGF to its specific cell-surface receptors elicits a pleitropic response in target cells. These effects include increased ion flux, enhanced phosphorylation of specific proteins, increased transcription of certain genes and accelerated DNA and protein synthesis (1-4). Although the mechanisms by which EGF mediates these processes have not been characterized, it is likely that some of these effects are regulated by the intrinsic tyrosine-specific protein kinase activity of the receptor (5,6). A number of other growth factor receptors, including those for insulin, IGF-I, CSF-I, PDGF

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and FGF, as well as several oncogene products of the src family, are also tyrosine-specific protein kinases (7). For this reason tyrosine phosphorylation is thought to play a role in growth factor-initiated signal transduction. It is important, therefore, to understand the nature of the coupling of EGF binding to stimulation of kinase activity. The human EGF receptor is an 1186-amino acid glycoprotein which contains an apparent 30-40 kDa of N-linked carbohydrate (8). We previously demonstrated that the initial receptor translation product does not possess high affinity EGF binding activity, and that this activity is acquired post-translationally in the endoplasmic reticulum (9, 10). The acquisition of EGF binding activity is inhibited by tunicamycin and deoxynojirimycin, indicating that this process requires N-linked core glycosylation and processing (10). In the present study we have investigated the development of autophosphorylation activity during post-translational processing of the EGF receptor. By employing both anti-phosphotyrosine and anti-EGF receptor antibodies we have shown that acquisition of autophosphorylation activity is not dependent upon N-linked core glycosylation.

MATERIALS AND METHODS

Protein A-Sepharose was obtained from Pharmacia P-L Biochemicals, and EGF Affi-Gel affinity matrix was prepared as described previously (9). Tunicamycin B2 was obtained from Boehringer Mannheim Biochemicals. Fluorosol was purchased from National Diagnostics. L-[^{35}S]Methionine (1100-1400 Ci/mmol) was from New England Nuclear. The preparation of affinity purified sheep anti-phosphotyrosine antibody was reported previously (11). Human A431 epidermoid carcinoma cells were maintained in monolayer culture and were labeled with [^{35}S]methionine as described previously (9). Tunicamycin B2 was used at 0.4 μg per ml since this homologue has a lower protein synthesis inhibitory capacity compared to the mixture of homologues usually employed. Immunoprecipitations were performed essentially as described previously (10). Briefly, 50 μl of Triton X-100 cell extract (equivalent to 50,000 cells) were treated with 100 μM ATP, 5 mM $MnCl_2$ and 10 μM Na_3VO_4 in a final volume of 100 μl. After 30 min at room temepratûre, phosphorylation was quenched with EDTA and the receptor was immunoprecipitated with either anti-EGF receptor or anti-phosphotyrosine antibody as described previously (9).

RESULTS AND DISCUSSION

Initially the effect of autophosphorylation (on tyrosine) on the electrophoretic mobility of the normal and aglyco forms of EGF receptor in SDS-poly-

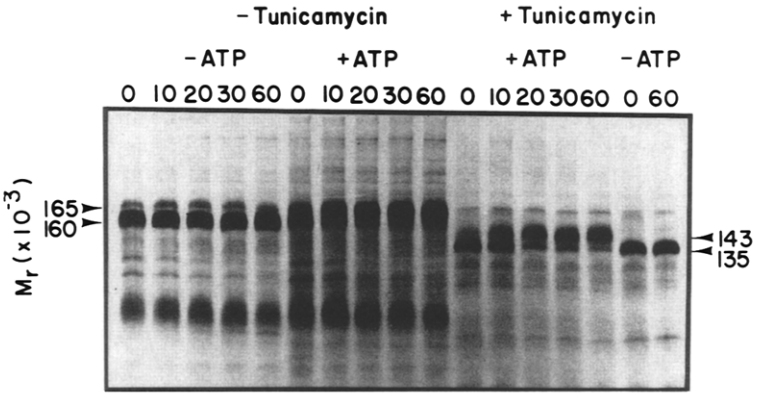

Fig. 1. Effect of autophosphorylation (by ATP) on the mobility by SDS-polyacrylamide gel electrophoresis of EGF receptor synthesized in the presence or absence of tunicamycin. Human A431 cells were pulse-labeled with [^{35}S]-methionine (0.2 mCi per ml) for 15 min either in the presence or absence of 0.4 μg per ml tunicamycin B2, and then were chased with medium containing unlabeled methionine for 0-60 min. Triton X-100 extracts of the cells, prepared at each chase time, were pretreated for 30 min either with (+ ATP) or without (- ATP) 100 μM ATP prior to immunoprecipitation with anti-EGF receptor antibody as described in Materials and Methods. Proteins were resolved by SDS-polyacrylamide (7.5%) gel electrophoresis under reducing conditions. The diffuse lower molecular weight band in the lanes without tunicamycin (- tunicamycin) is the truncated EGF receptor at M_r = 95-100,000.

acrylamide gels was determined. Human A431 epidermoid carcinoma cells were pulse-labeled with [^{35}S]methionine and then chased with unlabeled methionine for different periods of time up to 60 min either in the presence or absence of tunicamycin. As shown in Fig. 1 the fully glycosylated receptor, immunoprecipitated with anti-receptor antibody in the absence of ATP, exhibited a major and a minor band on SDS-polyacrylamide gels corresponding to 160 and 165 kDa, respectively. The apparent ratio of these two bands does not change during the 60 min chase period as we have reported previously (10). However, when incubated with ATP and then immunoprecipitated, the amount of receptor migrating at 165 kDa increases with increasing chase time and by 60 min it is the principal species. The observation that phosphorylated EGF receptor migrates more slowly upon SDS-polyacrylamide gel electrophoresis than the non-phosphorylated species has been reported previously (10, 12).

In the presence of tunicamycin which blocks N-linked glycosylation, a similar pattern is observed. In the absence of ATP, the aglyco-receptor migrates with an apparent M_r of 135,000 which remains unchanged throughout the 60-min chase. As in the presence of ATP, the apparent molecular weight is

shifted upward to 143 kDa and by 60 min of chase virtually none of the lower molecular weight species is detectable. It is interesting to note in Fig. 1 that the truncated form of the receptor (M_r = 95-100,000), which contains only the extracellular portion of the receptor and not the intracellular kinase domain (8, 13), does not exhibit this ATP-dependent shift.

In order to ascertain whether this shift in mobility was in fact due to a change in the phosphorylation state of the receptor, we employed an anti-phosphotyrosine antibody, which had previously been shown to immunoprecipitate the autophosphorylated (on tyrosine) form of the EGF receptor from A431 cells (10). The specificity of this antibody is demonstrated in Fig. 2, panel B, where it is shown that 5 mM phosphotyrosine completely blocks immunoprecipitation of the receptor, while phosphoserine and phosphothreonine have no effect. Furthermore, in panel C it is shown that in the absence of ATP, the receptor is not immunoprecipitated, indicating that it is not phosphorylated on tyrosine as isolated in the basal state. Fig. 2., panel A shows the results of

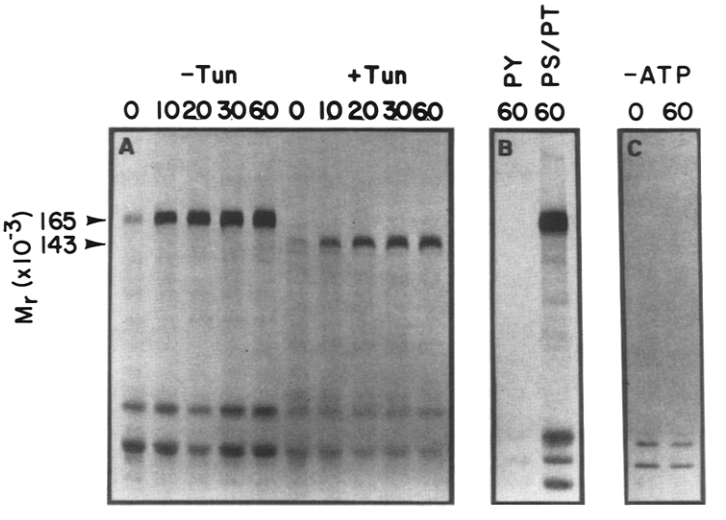

Fig. 2. Immunoprecipitation of phosphorylated EGF receptor (synthesized in the presence or absence of tunicamycin) with anti-phosphotyrosine antibody. (A) A431 cells were pulse-labeled with [^{35}S]methionine and then chased with unlabeled methionine in the presence or absence of 0.4 μg per ml of tunicamycin B2 as described in the legend to Fig. 1. Cell extracts were pretreated with ATP and then immunoprecipitated with anti-phosphotyrosine antibody. (B) Extracts of cells that had been chased for 60 min were immunoprecipitated in the presence of 5 mM phosphotyrosine (PY) or 2.5 mM phosphoserine and 2.5 mM phosphothreonine (PS/PT). (C) Extracts of cells pulsed for 0 and 60 min were immunoprecipitated in the absence of added ATP.

immunoprecipitating EGF receptor from A431 cells that had been pulsed with [^{35}S]methionine and chased for up to 60 min either in the presence or absence of 0.4 μg of tunicamycin per ml. With the anti-phosphotyrosine antibody, only the 143- and 165-kDa species (with and without tunicamycin, respectively) were immunoprecipitated, confirming their identification as the phosphorylated receptor species. Furthermore, the amount of receptor immunoprecipitable in the presence of ATP increased with chase time. This agrees with the time-dependent shift in apparent molecular weight of the receptor that was observed when the immunoprecipitation was performed with the anti-receptor antibody, and is consistent with the existence of a processing step by which the EGF receptor acquires either autophosphorylation activity or the capacity to undergo phosphorylation on tyrosine by unidentified kinases in the detergent extract. While the latter possibility cannot be rigorously excluded, it is unlikely since the only kinase demonstrated to phosphorylate the EGF receptor on tyrosine is the EGF receptor. Since the extent of phosphorylation in the basal state (in the absence of added EGF, but in the presence of exogeneous ATP) is already sufficient to allow virtually quantitative immunoprecipitation by the anti-phosphotyrosine antibody, it is not possible, using this technique, to demonstrate stimulation by EGF (10).

The results described above are presented quantitatively in Fig. 3A, which shows the amount of the phosphorylated 165 kDa species immunoprecipitated with either anti-EGF receptor antibody or antiphosphotyrosine antibody as a function of chase time, and in Fig. 3B, which shows the corresponding data for the aglyco species generated in the presence of tunicamycin. The time courses for both species as measured with either antibody appear quite similar. The apparently higher initial percentage of phosphorylated 165-kDa receptor measured by the anti-EGF receptor antibody (40% at zero min compared to approximately 20% for the other three time courses) is probably due to the difficulty in accurately resolving the two species in excising the gel slices.

In contrast to the results reported previously, which demonstrated the time course of acquisition of EGF binding activity (9, 10), the acquisition of autophosphorylation activity is independent of glycosylation and occurs more

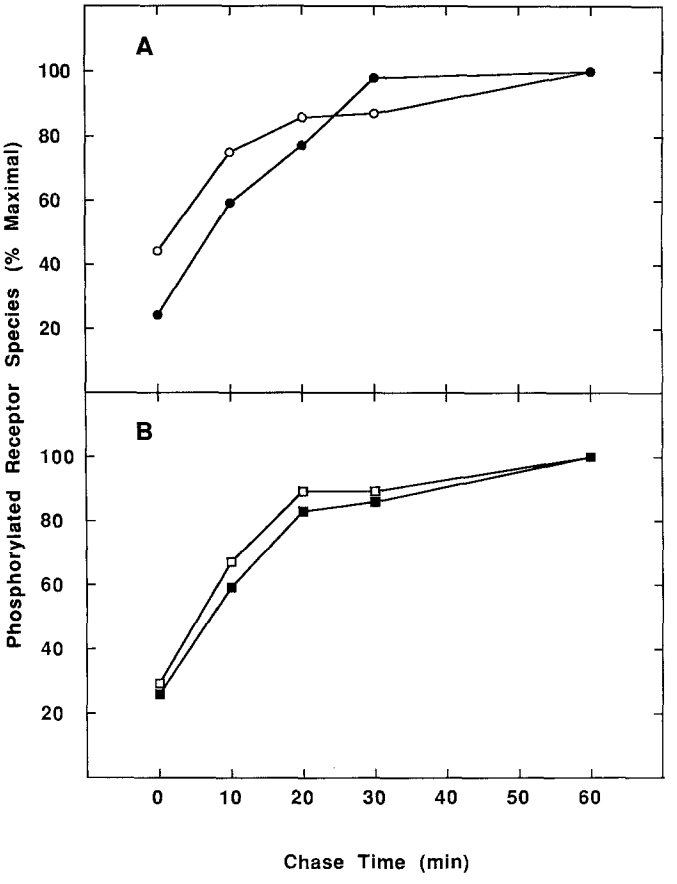

Fig. 3. Quantitation of post-translational acquisition of autophosphorylation activity. The data from Figs. 1 and 2 were quantitated by excising the EGF receptor bands from the gel and counting ^{35}S in Fluorosol. The results are presented as % of the maximally attained value. (A) Phosphorylated fully glycosylated EGF receptor (165 kDa, - tunicamycin) as measured with anti-EGF receptor antibody (-O-) and anti-phosphotyrosine antibody -●-). (B) Phosphorylated aglyco EGF receptor (143 kDa, + tunicamycin) as measured with anti-EGF receptor antibody (-□-) and anti-phosphotyrosine antibody (-■-).

rapidly ($t_{1/2}$ of approximately 10 min). Since newly-synthesized EGF receptor remains in the endoplasmic reticulum for a much longer period of time ($t_{1/2}$ = 90 min) (10), it is evident that both the acquisition of tyrosine kinase autophosphorylation activity ($t_{1/2}$ = 10-15 min) and of EGF binding activity ($t_{1/2}$ = 30-40 min) occur while the receptor resides in this organelle. The nature of the post-translational modifications by which the EGF receptor acquires the ability to undergo phosphorylation on tyrosine remains unclear. We previously suggested that the acquisition of EGF binding capacity might be related to a glycosylation dependent formation of a proper disulfide-bonded structure in the cysteine-rich regions of the extracellular domain of the

receptor (9,10). The intracellular tyrosine kinase domain of the receptor which contains the identified tyrosine autophosphorylation sites has only a few cysteine residues and presumably no sites of oligosaccharide addition. As most intracellular cysteine residues are not disulfide cross-linked it is not likely that the formation of this type of bond plays a role in the development of the tyrosine kinase autophosphorylation activity. Moreover, it is not likely that disulfide bond formation or interchange in the extracellular domain of the receptor is necessary for the acquisition of autophosphorylation activity, since v-erbB, which contains only the truncated intracellular region of the EGF receptor and which is the transforming protein of the erythroblastosis virus, possesses kinase activity in the absence of the disulfide cross-linked regulatory ligand binding domain (14). Biswas et al. (15) and Yarden and Schlessinger (16) have suggested that EGF stimulation of autophosphorylation activity may be associated with interconversion of the receptor between monomeric and dimeric states. We have investigated the sedimentation characteristics of the receptor between 0- and 120-min of chase (after an [^{35}S]methionine pulse) using rate zonal centrifugation in a sucrose gradient, and find that it sediments as the monomeric species (approximately 9S) at both the 0- and 120-min chase times (results not shown). This indicates that activation is not associated with changes in the state of aggregation of the receptor. Further investigation will be required to determine the mechanisms by which these post-translational modifications lead to acquisition of function by the EGF receptor.

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