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LIGAND-INDUCED POLYUBIQUITINATION OF RECEPTOR TYROSINE KINASES

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The platelet-derived growth factor β-receptor undergoes polyubiquitination as a consequence of ligand binding. In the present study, we have examined the ligand-induced receptor ubiquitination also in the other receptor tyrosine kinase (structurally different) subfamilies by immunoblotting with anti-ubiquitin antiserum. In addition to the platelet-derived growth factor α- and β-receptors, all the monomeric receptor tyrosine kinases examined, such as the receptors for epidermal growth factor (subfamily I), colony stimulating factor-1 (subfamily III), and fibroblast growth factor (subfamily IV), were found to be ubiquitinated after ligand stimulation. However, the insulin receptor (subfamily II), which is a tetrameric molecule, was not. These data suggest that the ligand-induced polyubiquitination of the receptor is a general phenomenon observed in most of the monomeric receptor tyrosine kinases.

Platelet-derived growth factor (PDGF) promotes the growth of mesenchymal cells in normal and pathological processes (1). Two types of the receptor for PDGF, designated α- and β-receptors, have been identified (2-4). They both belong to the receptor tyrosine kinase (RTK) subfamily III (5), consisting of five immunoglobulin-like repeats in the extracellular part and an intracellular tyrosine kinase domain, which is split into two parts by a non-catalytic insertion sequence, the kinase insert.

We have previously reported that the PDGF β-receptor undergoes polyubiquitination as a consequence of ligand binding (6), and have suggested that the

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The abbreviations used are: PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; CSF-1, colony stimulating factor-1; FGF, fibroblast growth factor; PAE, porcine aortic endothelial; CHO, chinese hamster ovary; WGA, wheat germ lectin.

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ligand-induced ubiquitination plays a negative regulatory role in mitogenic signaling of the PDGF β-receptor, possibly by promoting the efficient degradation of the ligand-activated receptor (7). Ubiquitin is present in eukaryotes and is a highly conserved 76-amino acid residue protein (8). Evidence supports the concept that ubiquitin conjugation to protein is implicated in ATP-dependent proteolytic pathways for short-lived proteins such as cyclins, Myc, Fos, and p53 (9).

In the present study, we report that, in addition to the PDGF β-receptor, other monomeric receptors belonging to different kinds of the RTK subfamily, including the epidermal growth factor (EGF) receptor (subfamily I), the PDGF α-receptor and the colony stimulating factor-1 (CSF-1) receptor (subfamily III), and the fibroblast growth factor (FGF) receptor (subfamily IV), are also polyubiquitinated after ligand stimulation, supporting the idea that the ligand-induced polyubiquitination of the receptor is a general phenomenon observed in most of the monomeric RTKs.

EXPERIMENTAL PROCEDURES

Cells: Porcine aortic endothelial (PAE) cells expressing the wild-type human PDGF α-receptor (10) or β-receptor (11) were prepared as described. PAE cells expressing the wild-type human FGF receptor-1 (FGFR-1) or a chimeric FGFR-1 where the FGFR-1 kinase insert was replaced with that of the PDGF β-receptor (FGFRchim), were prepared as described (12). Chinese hamster ovary (CHO) cells expressing the Ullrich-type (13) wild-type human insulin receptor was provided by Y. Kaburagi (14). The stably transfected PAE and CHO cells were cultured in Ham's F-12 medium (GIBCO) containing 10 % fetal bovine serum (GIBCO) and 200 mg/ml of the antibiotic G418 (GIBCO). NIH 3T3 cells expressing the wild-type human CSF-1 receptor or a mutant CSF-1 receptor where the carboxyl-terminally located Tyr-969 was changed to a phenylalanine residue (Y969F mutant), were provided by M. F. Roussel (15) and were cultured in Dulbecco's modified Eagles medium (GIBCO) containing 10 % fetal bovine serum and 200 mg/ml of G418. Human foreskin fibroblasts were obtained from a skin sample of a healthy 31-year-old male volunteer by an explant method (16) and were grown in Dulbecco's modified Eagles medium containing 10 % fetal bovine serum.
Antisera: The rabbit peptide antisera specifically reacting with the PDGF α-receptor (PDGFR-7) (4), the PDGF β-receptor (PDGFR-3) (17), and the FGFR-1 (18) were raised as described. The rabbit anti-CSF-1 receptor antiserum was provided by M. F. Roussel (15). The mouse monoclonal anti-EGF receptor antibody and the rabbit polyclonal anti-ubiquitin antiserum were purchased from Sigma. The mouse monoclonal anti-phosphotyrosine antibody (PY-20) was from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated sheep anti-mouse immunoglobulins and donkey anti-rabbit immunoglobulins were from Amersham.
Ligands: Recombinant human PDGF-BB, CSF-1, basic FGF and EGF were purchased from R & D Systems (Minneapolis, MN). Recombinant human insulin was from Upstate Biotechnology Inc. (Lake Placid, NY).
Immunoblotting: Immunoblotting was performed essentially as described by Mori *et al.* (6). After exposure of cells to the ligand, the cells were washed and lysed in the lysis buffer. The lysates were centrifuged and the supernatants were incubated with wheat germ lectin-Sepharose 6 MB (WGA-Sepharose) (Pharmacia) for 2 h at 4 °C. After washing with the lysis buffer, the beads were boiled for 3 min in the sample buffer containing 2 % 2-mercaptoethanol. The samples were separated by SDS-polyacrylamide gel electrophoresis and the proteins in the gel were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham). Blots were blocked, and incubated with the rabbit anti-receptor antisera (1:400 dilution), the monoclonal anti-EGF receptor antibody (1:2000 dilution), PY-20 (1:1000 dilution) or the anti-ubiquitin antiserum (1:1000 dilution). The blots were washed and then incubated with the peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulins (1:5000 dilution). After washing, sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham).

RESULTS

Fig. 1 shows a representative result of the receptor ubiquitination. PAE cells expressing the wild type human PDGF β-receptor were incubated with or without PDGF-BB, lysed, and a glycoprotein-enriched fraction of the cells, collected by adsorption to WGA-Sepharose, was separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. The blot was first probed with the β-receptor specific antiserum PDGFR-3. As shown in Fig. 1, a 190-kDa band, which corresponds to the mature form of the receptor, was detected in each *lane* (*lanes 1-2*). An upper smearing of the ligand-stimulated receptor band (*lane 2*) is due to ligand-induced receptor ubiquitination (6). The same blot was then stripped and reprobed with the anti-phosphotyrosine antibody PY-20 (Fig. 1, *lanes 3-4*). A 190-kDa band with a dense smearing was observed in the sample from the PDGF-BB-stimulated cells (*lane 4*), which corresponds to the activated and tyrosine-phosphorylated receptor. Finally, the blot was stripped again and reprobed with the anti-ubiquitin antiserum (Fig. 1, *lanes 5-6*). A broad band of more than 240 kDa was visualized after PDGF-BB stimulation (*lane 6*), which is composed of polyubiquitinated PDGF β-receptors (6). Thus, the immunoblotting assay sensitively reveals the presence of ubiquitinated receptors, and we employed this method to examine other RTKs with regard to the ligand-induced receptor ubiquitination.

First, we used PAE cells expressing the wild type human PDGF α-receptor. As shown in Fig. 2, a 170-kDa mature receptor band was observed by immunoblotting

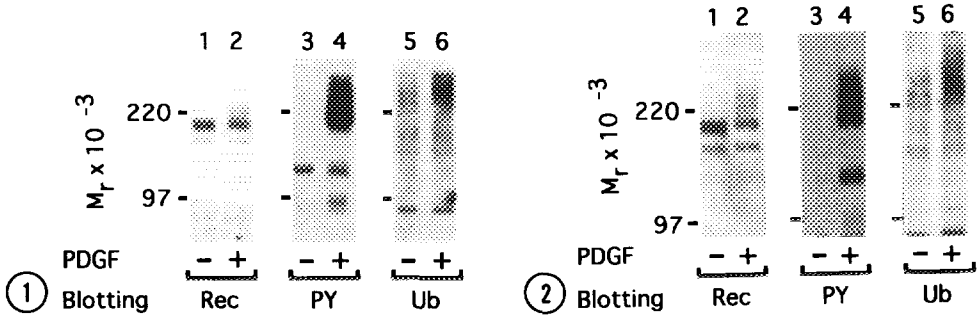

Fig. 1. PDGF-BB-induced ubiquitination of the PDGF β-receptor. PAE cells expressing the wild-type human PDGF β-receptor were incubated with (*lanes 2, 4* and *6*) or without (*lanes 1, 3* and *5*) 100 ng/ml PDGF-BB at 4 °C for 2 h. After incubation, the cells were lysed, and a glycoprotein-enriched fraction of the cells, collected by adsorption to WGA-Sepharose, was separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. The blot was probed with PDGFR-3 (*Rec*) (*lanes 1-2*), with PY-20 (*PY*) (*lanes 3-4*), and with the anti-ubiquitin antiserum (*Ub*) (*lanes 5-6*). Sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham). The relative migration positions of molecular weight standards (myosin, 220 kDa; phosphorylase *b*, 97.4 kDa) run in parallel are indicated.

Fig. 2. PDGF-BB-induced ubiquitination of the PDGF α-receptor. PAE cells expressing the wild-type human PDGF α-receptor were incubated with (*lanes 2, 4* and *6*) or without (*lanes 1, 3* and *5*) 100 ng/ml PDGF-BB at 4 °C for 2 h. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with PDGFR-7 (*Rec*) (*lanes 1-2*), with PY-20 (*PY*) (*lanes 3-4*), and with the anti-ubiquitin antiserum (*Ub*) (*lanes 5-6*).

with the anti-receptor antiserum PDGFR-7 (*lanes 1-2*). The pattern of smearing above the ligand-stimulated receptor band was similar to that observed for the PDGF β-receptor (compare Fig. 1, *lane 2* and Fig. 2, *lane 2*). Immunoblotting of the same blot with PY-20 (Fig. 2, *lanes 3-4*) detected a 170-kDa tyrosine-phosphorylated mature receptor band with a dense smearing, as expected (*lane 4*). Furthermore, the anti-ubiquitin blotting (Fig. 2, *lanes 5-6*) showed the existence of a broad band of more than 220 kDa after ligand stimulation (*lane 6*), which most likely corresponds to ubiquitinated PDGF α-receptors. Thus, the results indicate that the PDGF α-receptor is ubiquitinated after ligand stimulation.

Next, we examined the CSF-1 receptor which also belongs to the RTK subfamily III as the PDGF α- and β-receptors (5). NIH 3T3 cells expressing the wild-type human CSF-1 receptor or Y969F mutant receptor were stimulated with CSF-1, and the cells were subjected to the immunoblotting assay. As shown in Fig. 3, a 150-kDa band, which corresponds to the mature form of the receptor (15), was detected in each *lane* (*lanes 1-4*) by immunoblotting with the anti-receptor antiserum. Notably, the characteristic smearing of the ligand-stimulated receptor band was also observed for both the wild-type and Y969F mutant receptors (*lanes 2* and *4*). Immunoblotting with the anti-ubiquitin antiserum (Fig. 3, *lanes 5-8*) clearly detected a broad band of more than 220 kDa in the samples from the CSF-1-stimulated cells (*lanes 6* and *8*). The molecular size and intensity of the ubiquitinated receptor band was not different between the wild-type and Y969F mutant receptors. Thus, the results indicate that both the wild-type and Y969F mutant CSF-1 receptors are ubiquitinated with the same efficiency after ligand stimulation.

The FGF receptor belongs to the RTK subfamily IV, having a split intracellular tyrosine kinase domain with a relatively short kinase insert (14-17 amino acid residues) and three immunoglobulin-like repeats in the extracellular part (19). The structure looks similar to that of the RTK subfamily III, which prompted us to examine the ligand-induced receptor ubiquitination also in this family of the RTK. PAE cells expressing the wild-type human FGF receptor-1 (FGFR-1) or chimeric FGFR-1 (FGFRchim) were stimulated with basic FGF, and the cells were subjected to the immunoblotting assay. As shown in Fig. 4, two bands of 120 kDa and 150 kDa for the FGFR-1 (*lanes 1-2*) and 135 kDa and 165 kDa for the FGFRchim (*lanes 3-4*) were observed by immunoblotting with the anti-receptor antiserum. The smaller and the larger bands have previously been shown to correspond to the precursor and the mature forms of the receptors (12). Immunoblotting with PY-20 (Fig. 4, *lanes 5-8*) detected a tyrosine-phosphorylated mature receptor band of 150 kDa for the FGFR-1 (*lane 6*) and that of 165 kDa for the FGFRchim (*lane 8*) after basic FGF stimulation. A faint upper smearing of the ligand-stimulated mature receptor band was observed both for the FGFR-1 (*lane 6*) and FGFRchim (*lane 8*). Immunoblotting with the anti-ubiquitin antiserum (Fig. 4, *lanes 9-12*) could detect a broad band of more than 200 kDa for the FGFR-1 (*lane 10*) and that of more than 220 kDa for the FGFRchim (*lane 12*) in the samples from the ligand-stimulated cells. Intensity of the band was not different

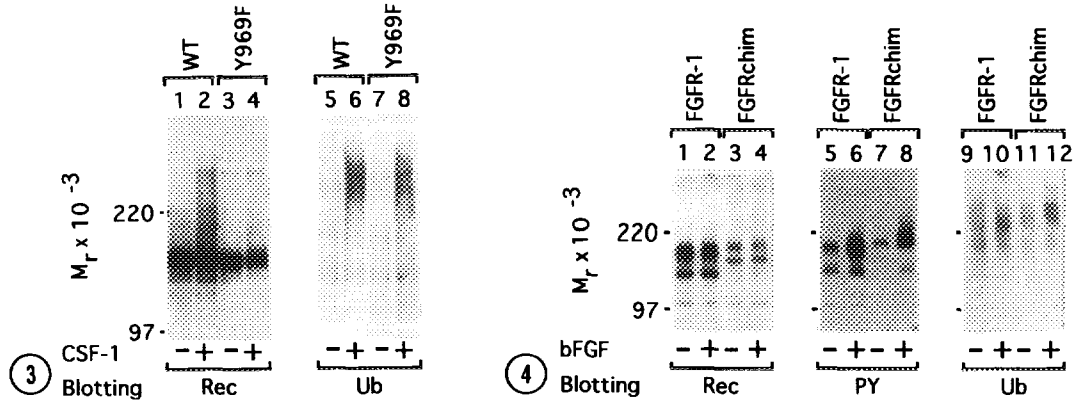

Fig. 3. CSF-1-induced ubiquitination of the CSF-1 receptor. NIH 3T3 cells expressing the wild-type (*WT*) or Y969F mutant human CSF-1 receptors were incubated with (*lanes 2, 4, 6* and *8*) or without (*lanes 1, 3, 5* and *7*) 100 ng/ml CSF-1 at 4 °C for 2 h. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with the anti-receptor antiserum (*Rec*) (*lanes 1-4*) and with the anti-ubiquitin antiserum (*Ub*) (*lanes 5-8*).

Fig. 4. Basic FGF-induced ubiquitination of the FGFR-1. PAE cells expressing the FGFR-1 or FGFRchim were incubated with (*lanes 2, 4, 6, 8, 10* and *12*) or without (*lanes 1, 3, 5, 7, 9* and *11*) 100 ng/ml basic FGF at 37 °C for 8 min. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with the anti-receptor antiserum (*Rec*) (*lanes 1-4*), with PY-20 (*PY*) (*lanes 5-8*), and with the anti-ubiquitin antiserum (*Ub*) (*lanes 9-12*).

between the FGFR-1 and FGFRchim, but that was apparenlty lower than those observed in the RTK subfamily III (see Figs. 1-3). Thus, we conclude that both the FGFR-1 and FGFRchim are ubiquitinated after ligand stimulation with the same efficiency, though the efficiency is low as compared with that of the RTK subfamily III.

Next we examined the EGF receptor which belongs to the RTK subfamily I. The member of this family possesses a continuous intracellular tyrosine kinase domain without any kinase insert. Human foreskin fibroblasts were stimulated with EGF, and the cells were subjected to the immunoblotting assay. As shown in Fig. 5, a 170-kDa band, which corresponds to the mature form of the EGF receptor, was observed in each lane (*lanes 1-2*) by immunoblotting with the anti-receptor antibody. Intensity of the EGF-stimulated receptor band (*lane 2*) was weaker as compared with that of the non-stimulated receptor band (*lane 1*), possibly due to ligand-induced smearing caused by ubiquitination of the receptor (ubiquitinated receptors have higher molecular sizes, thus their appearance will result in decrease in the amount of original molecular weight fraction of the receptor). Immunoblotting with PY-20 (Fig. 5, *lanes 3-4*) clearly demonstrated the presence of a tyrosine-phosphorylated mature receptor band accompanied by a dense upper smearing in the sample from the EGF-stimulated cells (*lane 4*). Immunoblotting with the anti-ubiquitin antiserum (Fig. 5, *lanes 5-6*) detected a broad band of more than 220 kDa only in the sample from the EGF-stimulated cells, as expected (*lane 6*). Thus, the results indicate that the EGF receptor is ubiquitinated after ligand stimulation.

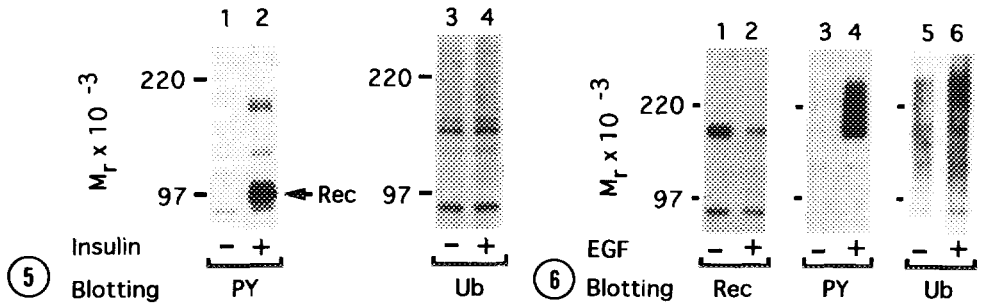

Fig. 5. EGF-induced ubiquitination of the EGF receptor. Human foreskin fibroblasts were incubated with (*lanes 2, 4* and *6*) or without (*lanes 1, 3* and *5*) 100 ng/ml EGF at 4 °C for 2 h. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with the anti-EGF receptor antibody (*Rec*) (*lanes 1-2*), with PY-20 (*PY*) (*lanes 3-4*), and with the anti-ubiquitin antiserum (*Ub*) (*lanes 5-6*).

Fig. 6. The insulin receptor is not ubiquitinated after ligand stimulation. CHO cells expressing the wild-type human insulin receptor were incubated with (*lanes 2* and *4*) or without (*lanes 1* and *3*) 5 mg/ml insulin at 37 °C for 8 min. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with PY-20 (*PY*) (*lanes 1-2*) and with the anti-ubiquitin antiserum (*Ub*) (*lanes 3-4*). The migration position of the β-subunit of the insulin receptor (*Rec*) is indicated by an arrowhead.

Finally, we examined the insulin receptor which belongs to the RTK subfamily II. The receptor is a tetrameric molecule composed of a pair of extracellular α-subunits and transmembrane β-subunits, which are extracellularly connected to each other by disulfide bonds. CHO cells expressing the wild-type human insulin receptor were stimulated with insulin, and the cells were subjected to the immunoblotting assay. As shown in Fig. 6, immunoblotting with PY-20 detected a 95-kDa clear band with no apparent smearing in the sample from the insulin-stimulated cells (*lane 2*). The band has been shown to correspond to tyrosine-phosphorylated β-subunits of the insulin receptor (14). The same blot was stripped and reprobed with the anti-ubiquitin antiserum. As shown in Fig. 6, no specific band was observed even when the blot was exposed to the film for extremely prolonged time (*lanes 3-4*). Thus, we conclude that the insulin receptor is not ubiquitinated after ligand stimulation.

DISCUSSION

In the present study we demonstrate that, with the exception of the tetrameric insulin receptor, all the monomeric RTKs examined, namely, the EGF receptor, PDGF α- and β-receptors, CSF-1 receptor, and FGFR-1, are ubiquitinated after ligand stimulation. The band detected by the immunoblotting with the anti-ubiquitin antiserum was broad, suggesting that the receptors are polyubiquitinated, since polyubiquitination will create different molecular-weight components according to the number of the attached ubiquitin molecules (6). Miyazawa *et al.* (20) recently reported that the c-*kit*-encoded protein receptor, which also belongs to the RTK subfamily III as the PDGF

and CSF-1 receptors, is polyubiquitinated after ligand stimulation. Thus, the data, together with our present data, strongly support the idea that the ligand-induced polyubiquitination of the receptor is a general phenomenon observed in most of the monomeric RTKs.

We have observed that a mutant PDGF β-receptor in which the carboxyl-terminally located Tyr-1009 and Tyr-1021, which are autophosphorylation sites, were simultaneously changed to phenylalanine residues by site-directed mutagenesis (Y1009/1021F mutant) shows an amplified mitogenic activity, possibly due to increased half life of the ligand-activated receptor caused by decrease in the efficiency of the receptor ubiquitination (less than 15 % of the wild-type receptor) (7). Roussel *et al.* (21) have reported that a mutation of the CSF-1 receptor which changed the carboxyl-terminally located Tyr-969 to a phenylalanine residue (Y969F mutant), although not in itself sufficient to induce transformation, activates the oncogenic potential of the CSF-1 receptor in association with an endogenously expressed CSF-1. These analogous findings of a negative regulation on mitogenesis exerted by the carboxyl-terminally located tyrosine residue(s) in the PDGF β-receptor and the CSF-1 receptor prompted us to examine the ligand-induced ubiquitination also in the Y969F mutant CSF-1 receptor. As shown in Fig. 3, both the wild-type and Y969F mutant CSF-1 receptors were ubiquitinated with the same efficiency after ligand stimulation, indicating that a mechanism responsible for the negative regulation by Tyr-969 of the CSF-1 receptor is not related to the receptor ubiquitination.

The efficiency of ubiquitination of the FGFR-1 was apparently lower than those of the PDGF, CSF-1 and EGF receptors (Figs. 1-5). The difference in the efficiency of ubiquitination is most likely due to some structural difference(s) in the intracellular part of the receptors. We have previously shown that the replacement of the FGFR-1 kinase insert with that of the PDGF β-receptor results in transfer of certain β-receptor specific signaling properties to the chimeric molecule; the FGFRchim mediates membrane ruffling and chemotaxis through activation of phosphatidylinositol 3' kinase, which is not mediated by the FGFR-1 (22). Therefore, it was of interest to examine the ligand-induced ubiquitination of the FGFRchim with the aim of evaluating the role of the PDGF β-receptor kinase insert in the receptor ubiquitination. As shown in Fig. 4, the efficiency of ubiquitination of the FGFRchim was not increased as compared with that of the FGFR-1, suggesting that the kinase insert of the PDGF β-receptor does not play a significant role in the ubiquitination process.

The present result that the insulin receptor was not ubiquitinated after ligand stimulation (Fig. 6) is very interesting considering the fact that the other monomeric RTKs examined to date by us were all ubiquitinated. If the ligand-induced ubiquitination is requisite for some specific function of the RTKs, it is conceivable that, instead of the insulin receptor itself, another molecule which is involved in the signal transduction by the insulin receptor should be ubiquitinated after activation of the insulin receptor. Our future studies will be aimed at exploring the possibility.

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