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Kinetics of phosphorylation of the SH2-containing domain of phospholipase Cγ1 by the epidermal growth factor receptor

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Abstract The kinetics of the EGF receptor (EGFR) autophosphorylation and of the phosphorylation by EGFR of a fusion protein (Fp(SH2)) derived from PLC-γ_1 with two SH2 domains were studied employing purfied EGFR or membrane-bound preparations of native and truncated EGFR. With varied ATP concentrations both reactions yielded Michaelis–Menten kinetics. K_{ATP} for autophosphorylation was 0.35 μM and for Fp(SH2) phosphorylation 1.35 μM. With Fp(SH2) as variable, the velocity curves for substrate phosphorylation by the various EGFR preparations were sigmoidal, reached peaks at 0.45 μM Fp(SH2) and were followed by drops to zero velocities at about 1.0 μM Fp(SH2). We conclude that (a) our data support the concept that receptor autophosphorylation is a prerequisite for the interactions between EGFR and the substrate's SH2-domains and their eventual phosphorylation by the receptor, and (b) the interactions between EGFR and the physiological substrate seem to involve mechanisms which allow the substrate to act as an on–off switch in the subsequent substrate phosphorylation reaction.

Key words: Receptor; EGF receptor; Phospholipase Cγ1; SH2 domain; Tyrosine kinase; EGFR kinetics

1. Introduction

The epidermal growth factor (EGF) receptor (EGFR), like many proto-oncogene products, belongs to a large family of protein tyrosine kinases (PTKs) which play an important role in signal transduction [1,2], a process which involves the conversion of an extracellular input signal at the membranal level into an intracellular signal which eventually affects gene expression at the nuclear level. The activity of the EGFR tyrosine kinase (TK) domain, which is similar to that of many other oncogene products [3], is controlled by EGF and plays a major role in the regulation of cell proliferation. EGFR undergoes autophosphorylation on five high affinity tyrosine residues localized to the C-terminus of the receptor. The receptor also phosphorylates external substrates. These two processes are markedly enhanced by EGF both in vivo and in vitro. This ligand-induced receptor activation requires oligomerization (mostly dimerization) of the EGF receptor which brings about the process of intermolecular trans-autophosphorylation [4]. Although EGFR can phosphorylate various protein substrates in vitro, it is well established that the physiological intracellular substrates of the EGFR are proteins which contain the Src homology 2 domain (SH2 domain) [5]. Thus, for example, the EGF-dependent phosphorylation of PLC-γ_1 by the EGFR [6] involves the high affinity binding of the enzyme to autophosphorylated receptor molecules through interactions of the SH2 domains of PLC-γ_1 with autophosphorylated sites on the EGFR including the 5 highly reactive tyrosines of the C-terminus (Y992, Y1068, Y1086, Y1148 and Y1173) [7].

Based on extensive and detailed kinetic studies with EGFR isolated from A431 cells with a synthetic copolimer (poly-$Glu_6Ala_3Tyr_1$, GAT), angiotensin II and ATP as substrates, we have recently concluded that the TK reaction of the EGFR follows a Sequential Rapid Equilibrium Random Bi-Bi mechanism [8,9]. In view of recent knowledge concerning the involvement of SH2 domains in the interactions of EGFR with its physiological substrates, we decided to study the kinetics of phosphorylation of PLC-γ_1 constructs which posses the SH2 domains by EGFR.

2. Materials and methods

2.1. Materials

Mouse EGF (tissue culture grade) was obtained from Serotec, Oxford, [γ-^{32}P]ATP was purchased from Amersham, UK, ATP was purchased from Böhringer (Mannheim). Agarose-immobilized wheat germ agglutinin was obtained from Biomakor (Rehovoth, Israel). Glutathione-agarose beads and all other biochemicals were obtained from Sigma (St. Louis, MO, USA) and were of the highest purity available; water was deionized by the Barnsted Nanopure system. Monoclonal Ab108, a gift from Dr. J. Schlessinger, New York University Medical School and Dr. A. Zilberstein, Rhone Poulenc Rorer. CD63 cells [10] and CD126 cells [11] were a gift from Drs. J. Schlessinger and A. Ulrich and the SH2/SH3-containing PLC-γ_1-pGEX-3X plasmid were a generous gift from Dr. J. Schlessinger.

2.2. Preparation of EGFR

EGFR was purified from human epidermoid carcinoma A431 cells as described elsewhere [8,12] and stored at −70°C. When subjected to immunoblot with anti-EGFR antibodies the purified receptor appeared as one band (170 kDa, not shown). Also, when subjected to treatment with ATP and EGF and then to immunoblot using antiphosphotyrosine antibodies, a single 170 kDa band was observed (not shown).

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Abbreviations: BMN, benzenemalononitrile; CD63, NIH 3T3 cells expressing an active EGFR mutant lacking 63 amino acids including Y1148 and Y1173; CD126, NIH3T3 cells expressing a truncated EGFR lacking 126 amino acid including Y1068, Y1086, Y1148 and Y1173; DTT, dithiothreitol; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; EGFR, EGF receptor; EGFR-P, pre-phosphorylated EGF receptor; Fp(SH_2), GST fusion protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HER14, native NIH3T3/HER14 cells; GST, glutathione S-transferase; MES, 2-[Morpholino]-ethanesulfonic acid; SH2 domain, Src homology 2 domain ; TCA, trichloroacetic acid; PTK, protein tyrosine kinase catalytic subunit; TK, tyrosine kinase.

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2.3. Preparation of cell membranes

NIH3T3/HER14 cells [12] which express approximately 300,000 native EGFR molecules per cell, NIH3T3/CD63 cells (NIH 3T3 cells expressing approximately 300,000 molecules per cell of an active EGFR mutant lacking 63 amino acids from the C-terminus including the 2 tyrosine autophosphorylation sites Y1148 and Y1173) and NIH3T3/CD126 cells (which express a truncated EGFR lacking 126 amino acids from the C-terminus including the 4 tyrosine autophosphorylation sites Y1068, Y1086, Y1148 and Y1173) were grown to confluence in Dulbecco modified Eagle's medium – 50 mg/ml penicillin – 10% fetal calf serum [10,11,13]. Membranes were prepared as described elsewhere [12].

2.4. Preparation of PLCγ_1-SH$_2$ domain

A DNA insert corresponding to the boundaries of the SH2/SH3 sequences of human PLC-γ_1 containing the two phosphorylation sites Y771 and Y783 and which is ligated into pGEX-3X bacterial expression plasmid was a gift of Dr. J. Schlessinger. *E. coli* were transfected with the recombinant plasmid and after confirming that the bacterial transformants contained the insert, the glutathione S-transferase (GST) fusion protein was expressed by induction with 1 mM isopropyl β-D-thiogalactopyranoside. The GST fusion protein (Fp(SH$_2$)) was isolated from bacterial lysates by affinity chromatography with glutathione-agarose beads and elution with 10 mM reduced glutathione [14,15]. Protein concentrations were determined by the methods of Scultz et al. [16] and Peterson [17].

2.5. Assay of EGFR-TK activity

Assays were performed in nonsterile culture plates with 96 conical wells [9,12]. Assay mixtures contained [γ-^{32}P]ATP (2–5 μCi), fixed concentrations of Fp(SH$_2$) and increasing concentrations of ATP, or vice versa, 50 mM Tris-MES, 2 mM $MnAc_2$, 10 mM $MgAc_2$, 0.1 mM Na_3VO_4, 2 mM glutathione, pH 8.0, in total volumes of 20 μl. EGFR or membrane preparations were pre-activated immediately prior to use by incubation for 30 min at 4°C in presence of 800 nM EGF and 10 mM $MgAc_2$, 2 mM $MnAc_2$, 100 μM Na_3VO_4 in 50 mM TM, pH 8.0. Reactions were initiated by the simultaneous addition of cold, activated EGFR to series of 8 wells with the aid of a multipipetor (5 μl/well). In some experiments EGFR was preactivated in the presence of 5.0 μM ATP and 50 mM Tris-MES, 2 mM $MnAc_2$, 10 mM $MgAc_2$, 0.1 mM Na_3VO_4, pH 8.0 in order to induce autophosphorylation of the receptor prior to its addition to reaction mixtures which contained Fp(SH$_2$). After incubation at 22°C for time intervals indicated in the text, the reactions were stopped by the addition with the aid of the multipipetor of 8 μl aliquots of hot (65°C) 4-fold concentrated sample buffer. The EGFR and the Fp(SH$_2$) substrate were fractionated by SDS-PAGE, the gels were placed on Whatmann 3MM filter paper, dried and autoradiographed. Films were superimposed on corresponding gels and areas corresponding to phosphorylated protein bands were cut out and their radioactivity was determined by Cerenkov counting in a Packard CD1600 scintillation counter.

3. Results and discussion

The phosphorylation of the fusion protein by purified EGFR and receptor autophosphorylation were compared with the phosphorylation of the fusion protein by native and by truncated versions of EGFR (CD63 and CD126) present in membrane preparations and their autophosphorylation. In these studies ATP concentrations were varied while maintaining the concentration of the fusion protein constant (0.45 μM). As can be seen in Fig. 1A–D, all reactions followed Michaelis–Menten kinetics. The various apparent dissociation constants (i.e. the

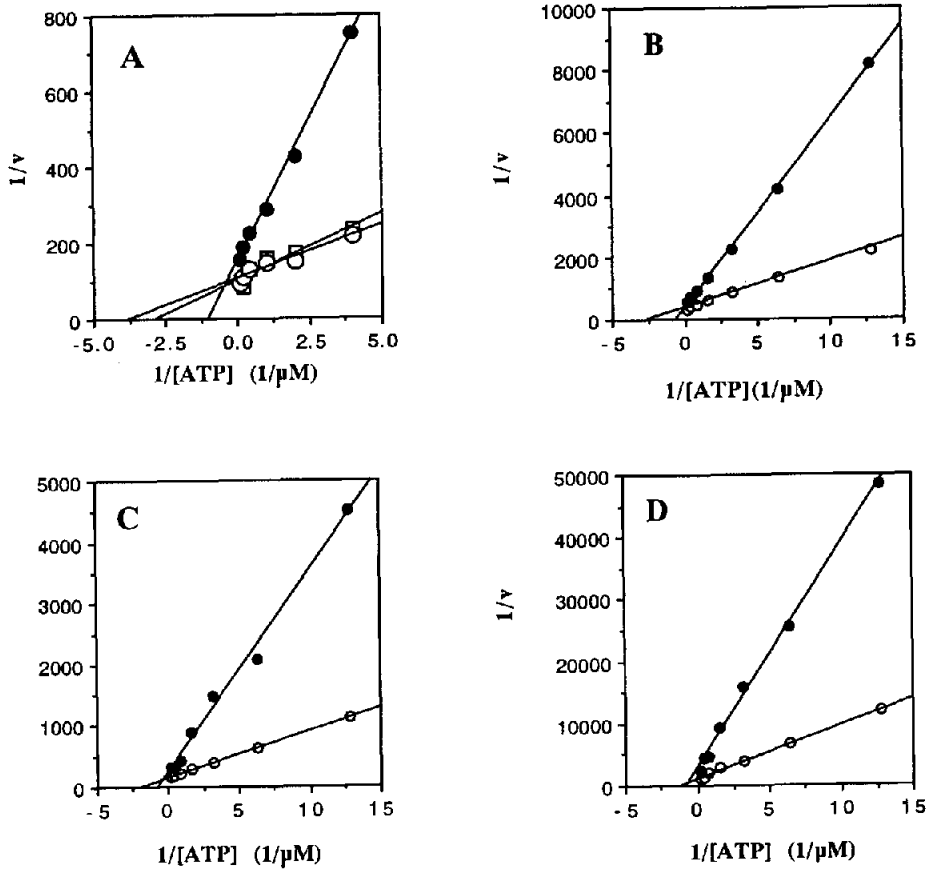

Fig. 1. Autophosphorylation and the phosphorylation of Fp(SH2) by purified EGFR and by purified membrane preparations at varying ATP concentrations. Autophosphorylation of EGFR at (○) 0 μM Fp(SH2), or (□) 0.45 μM Fp(SH2), and (●) phosphorylation of 0.45 μM Fp(SH2) by (A) purified EGFR, (B) membranes prepared from DHER cells, (C) membranes prepared from CD63 cells and (D) membranes prepared from CD126 cells.

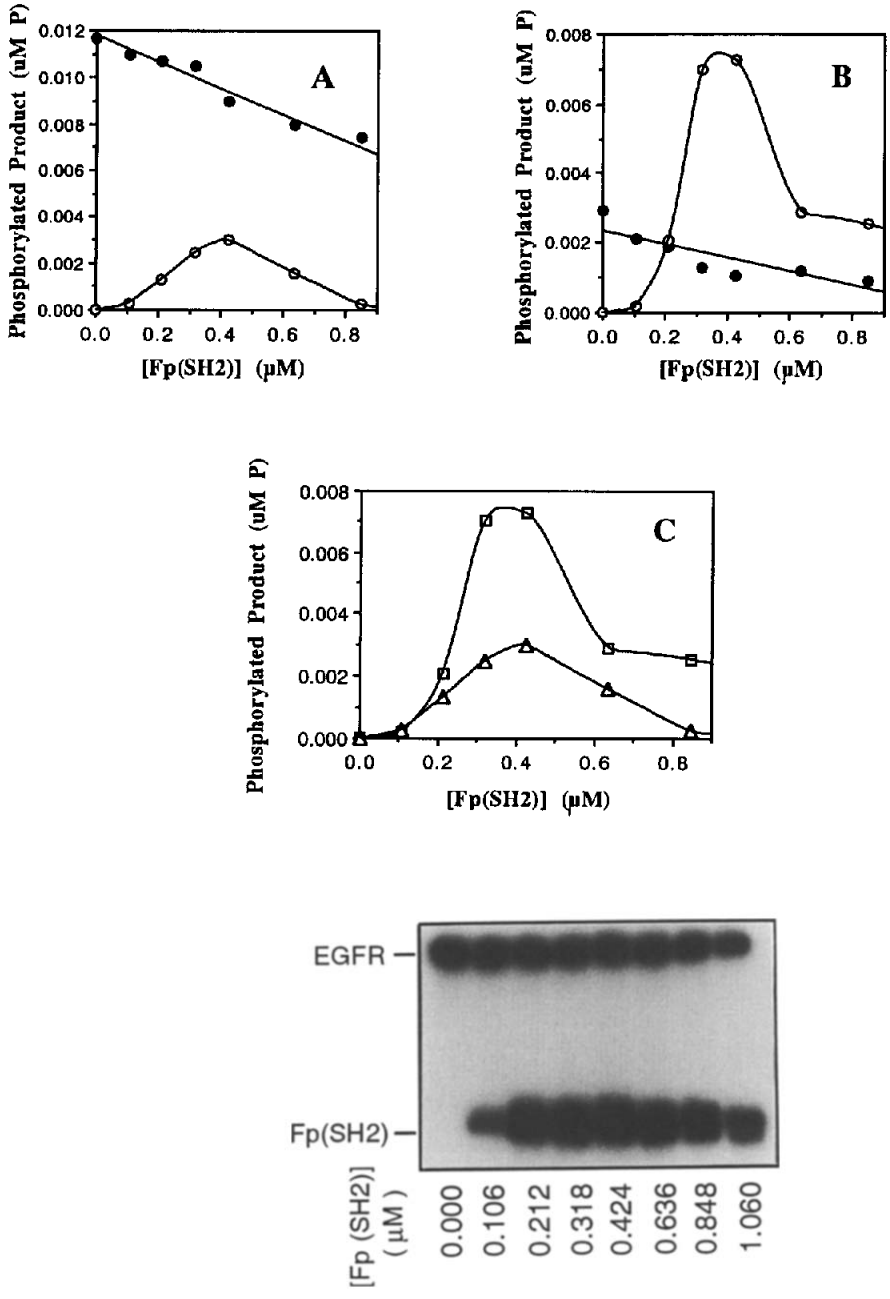

Fig. 2. Autophosphorylation and phosphorylation of Fp(SH2) at varying Fp(SH2) concentrations. A. (●) EGFR autophosphorylation, (○) phosphorylation of Fp(SH2) at 1.0 μM final [ATP]. Reactions were stopped after 15 s. B. Experimental conditions were as in A, except that EGFR was pre-phosphorylated. (●) EGFR-P autophosphorylation, (○) phosphorylation of Fp(SH2). C. A comparison between the phosphorylation of Fp(SH2) by (△) EGFR and (□) pre-phosphorylated EGFR. D. A characteristic autoradiogram showing EGFR autophosphorylation and the phosphorylation of Fp(SH2) at the indicated micromolar concentrations of Fp(SH2).

apparent K_m) for ATP ($K_{ATP(EGFR)}$ or $K_{ATP(Fp(SH2))}$) computed from these data as described elsewhere [9] are shown in Table 1. $K_{ATP(EGFR)}$ for the purified EGFR and the membranous native EGFR were quite similar. Fp(SH2) slightly increased the apparent value of the dissociation constant. Similar $K_{ATP(EGFR)}$ values were observed at higher Fp(SH2) concentrations and at longer reaction times (data not shown). The apparent $K_{ATP(EGFR)}$ for the CD126 was higher than for CD63 and the latter was higher than for the native receptor. The apparent $K_{ATP(Fp(SH2))}$ computed from the plots of the phosphorylation of the fusion protein is similar in all four types of EGFR studied (Table 1).

The autophosphorylation of EGFR and the phosphorylation of Fp(SH2) by EGFR over a short period of 15 s are shown in Fig. 2A. While the autophosphorylation of EGFR decreased with increasing Fp(SH2) concentrations, undoubtedly due to competition for the receptor's catalytic site, the phosphorylation of the fusion protein increased sigmoidally with increasing substrate concentrations, but after reaching a peak at about 0.45 μM Fp(SH2), the reaction rates decreased to practically

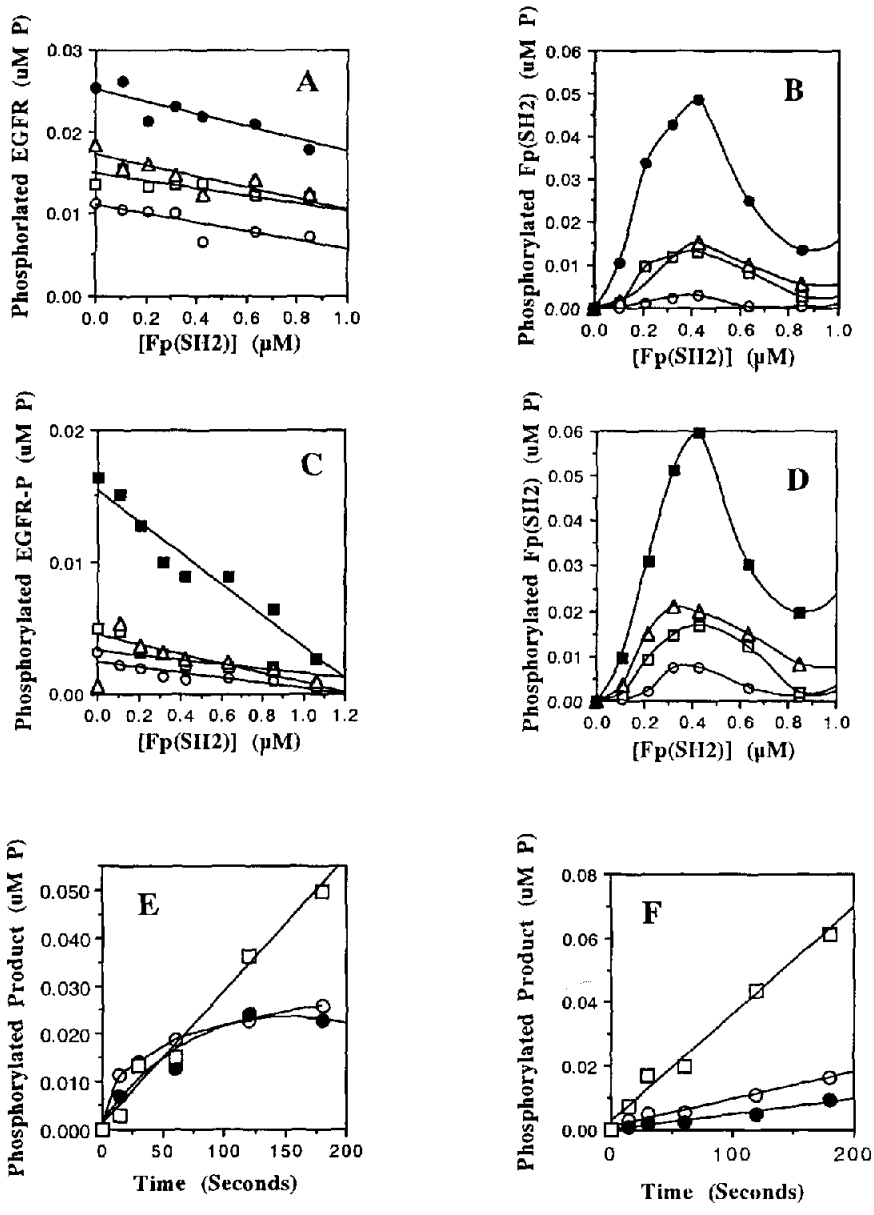

Fig. 3. Autophosphorylation and the phosphorylation of Fp(SH2) by EGFR and EGFR-P. Autophosphorylation of (A) EGFR and (C) EGFR-P and the phosphorylation of Fp(SH2) by (B) EGFR or (D) EGFR-P for: (○) 15 s, (□) 30 s, (△) 60 s, or (●) 120 s at final [ATP] of 1.0 μM. E. The autophosphorylation of EGFR (○) in the absence and (●) in the presence of 0.45 μM Fp(SH2 and (□) the phosphorylation of 0.45 μM Fp(SH2) by EGFR. F. The autophosphorylation of EGFR-P (○) in the absence and (●) the presence of 0.45 μM Fp(SH2 and (□) the phosphorylation of 0.45 μM Fp(SH2) by EGFR-P.

zero. Similar results were observed with EGFR which was allowed to undergo extensive autophosphorylation (EGFR-P) prior to the addition of Fp(SH2) (Fig. 2B). Similar results were observed at ATP concentrations smaller and higher than those employed in these experiments (1 μM; data not shown). It is interesting to note that the phosphorylation of Fp(SH2) by EGFR-P reached a peak at a far higher value than the phosphorylation by EGFR which presumably was unphosphorylated prior to its transfer to the reaction media (Fig. 2C). These results suggest that receptor autophosphorylation is a prerequisite for the interactions between EGFR and substrates containing SH2-domains and their eventual phosphorylation by the receptor as concluded by others [5].

In similar experiments, Fp(SH2) was phosphorylated by

Table 1
Values of ATP·EGFR or ATP·Fp(SH2) apparent dissociation constants (K_{ATP}) obtained from kinetic studies.

Type of receptor	Type of reaction	
	Receptor auto-phosphorylation	Phosphorylation of Fp(SH2)
EGFR[1] (no Fp(SH2))	0.27[2]	
EGFR[1]	0.35[3]	1.0
Native EGFR[4]	0.41[3]	1.47
CD63[4]	0.52[3]	1.40
CD126[4]	0.80[3]	1.34

[1] Purified EGFR; 20 s reaction time.
[2] Values are in μM.
[3] Reactions were performed at 0.45 μM Fp(SH2).
[4] Native or truncated EGFR in membrane preparations; 30 s reaction time.

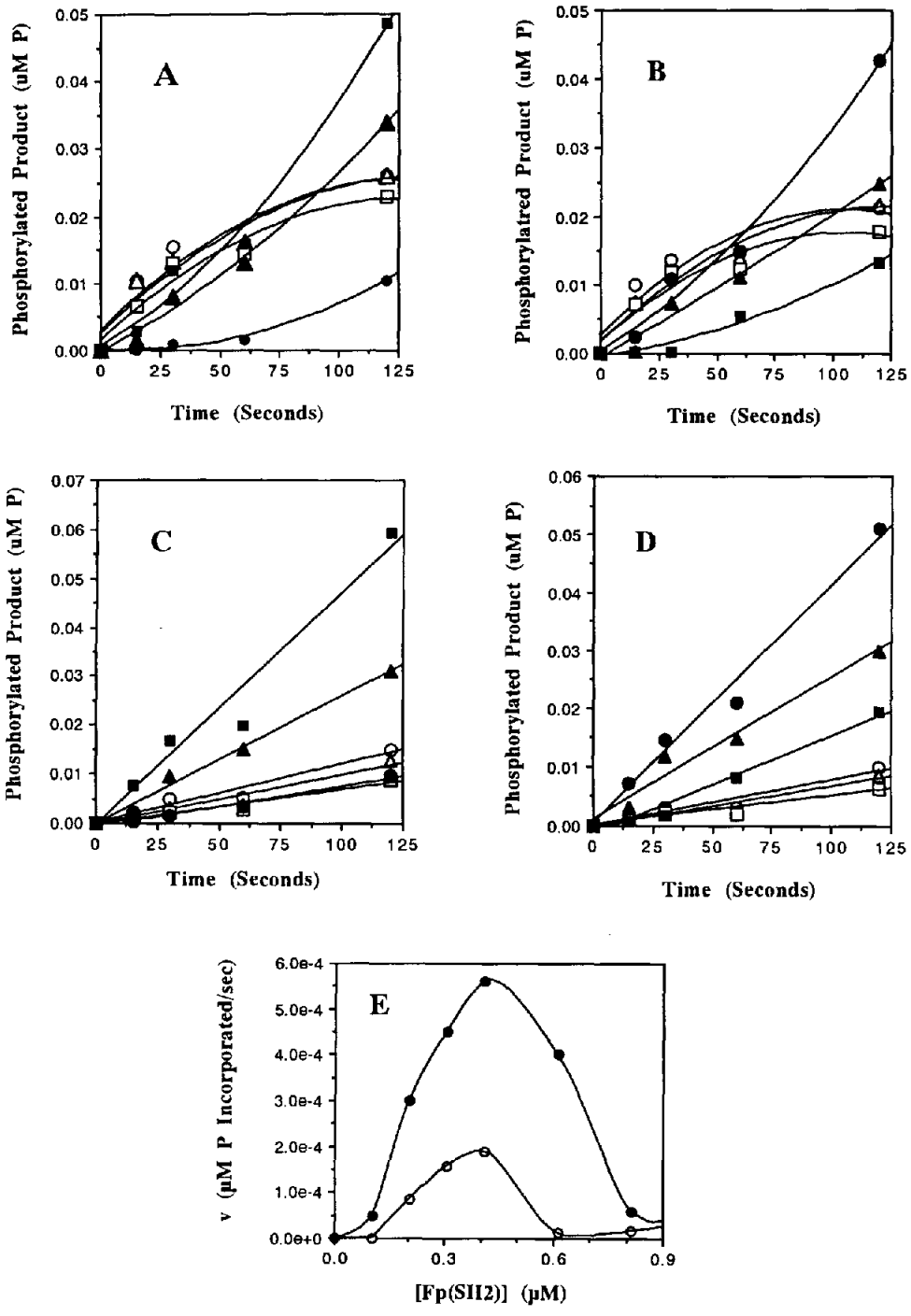

Fig. 4. Rate of EGFR and EGFR-P autophosphorylation and Fp(SH2) phosphorylation. In (A), (B), (C) and (D) autophosphorylation is indicated by open symbols and the phosphorylation of Fp(SH2) by filled symbols. (A) Phosphorylation by EGFR and (C) Phosphorylation by EGFR-P at [FpSH2)] of: (○) and (●) 0.106 μM; (△) and (▲) 0.212 μM; (□) and (■) 0.424 μM. (C) Phosphorylation by EGFR and (D) phosphorylation by EGFR-P at [FpSH2)] of: (○) and (●) 0.318 μM; (△) and (▲) 0.612 μM; (□) and (■) 0.816 μM. E. Rates of phosphorylation of Fp(SH2) by (○) EGFR and by (●) EGFR-P at final [ATP] of 1.0 μM and varying Fp(SH2) concentrations.

EGFR or by pre-phosphorylated EGFR (EGFR-P) at final [ATP] = 1.0 μM over time periods ranging from 0 s to 120 s. At all time intervals the autophosphorylation of either EGFR or EGFR-P decreased linearly with increasing Fp(SH2) concentrations (Fig. 3A and C, respectively), whereas the phosphorylation of Fp(SH2) increased sigmoidally and reached peaks at 0.45 μM Fp(SH2) whether phosphorylated by EGFR or by EGFR-P (Fig. 3B and D, respectively). Preliminary experiments have shown that the autophosphorylation of EGFR time curve is hyperbolic (Fig. 3E): there is a rapid initial rate of autophosphorylation of EGFR, which represents the phosphorylation of the receptor's highly reactive tyrosine residues that

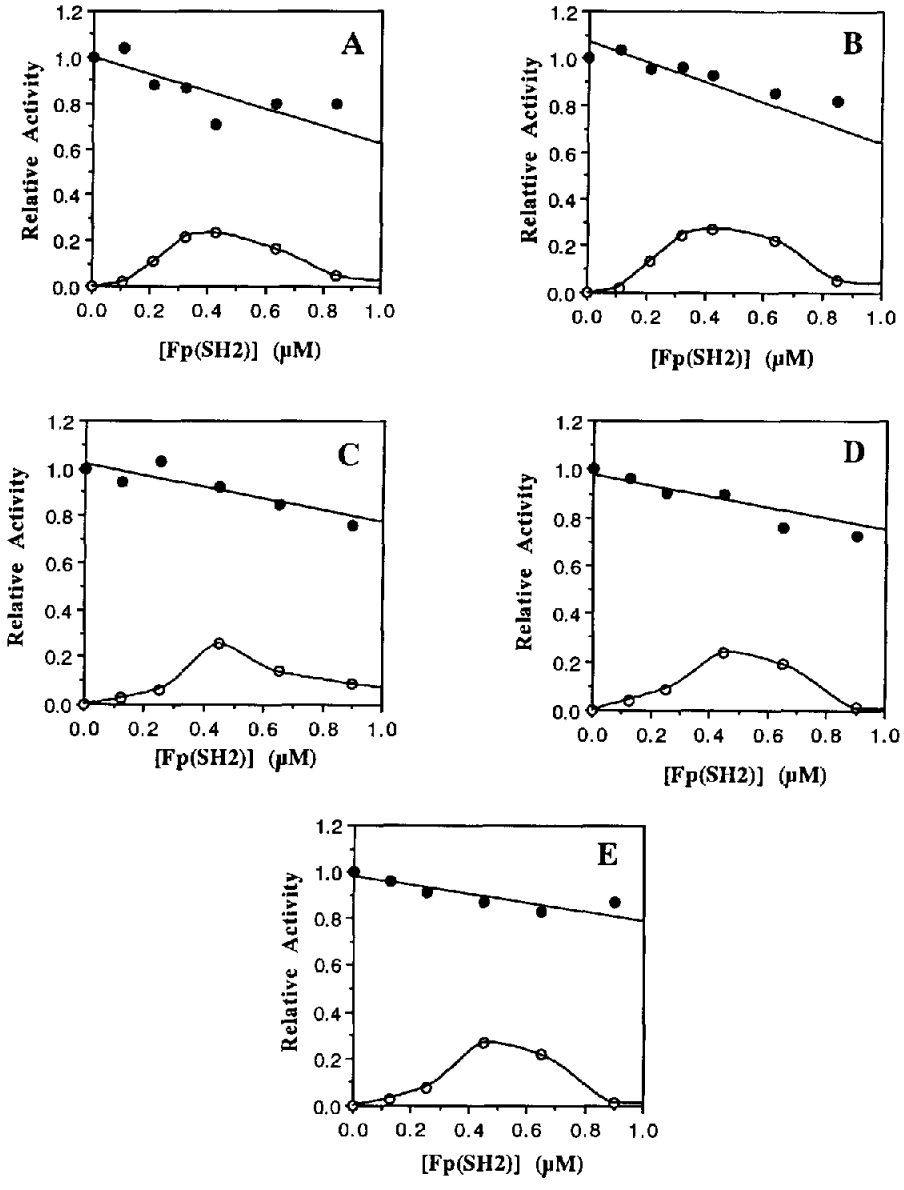

Fig. 5. Autophosphorylation and phosphorylation of Fp(SH2) by purified EGFR and by purified membrane preparations at varying Fp(SH2) concentrations. (●) autophosphorylation of EGFR and (○) phosphorylation of Fp(SH2) by (A) purified EGFR preincubated in the absence of EGF; (B) purified EGFR preincubated in the presence of EGF; (C) membranes prepared from DHER cells; (D) membranes prepared from CD63 cells; and (E) membranes prepared from CD126 cells.

are involved in the EGFR:Fp(SH2) interactions [4,10]; after about 60 s, the rate of autophosphorylation tends to level off and possibly involves the phosphorylation of low affinity slowly phosphorylated tyrosine residues. Fp(SH2) competes with the receptor's autophosphorylation sites for binding to the receptor's active center and thus causes a decrease in the initial rate of autophosphorylation of EGFR. The phosphorylation of the fusion protein, Fig. 3E, increases with time to reach far higher levels than the receptor autophosphorylation at longer time intervals. Fig. 3F shows that despite the relatively long preincubation of the receptor at ATP concentrations almost 20-fold greater the autophosphorylation K_{ATP} (Table 1), there was some further linear increase in the autophosphorylation of EGFR-P and that Fp(SH2) caused some inhibition of this autophosphorylation while the phosphorylation of Fp(SH2) also increased linearly with time. The above results suggest that the reactions that are relevant for EGFR:Fp(SH2) interactions must occur shortly after the initiation of the autophosphorylation and Fp(SH2) phosphorylation reactions.

In view of the above, experiments similar to those shown in Fig. 3A–D were repeated at incubation times of 0–120 s and the data are shown in Fig. 4A–E. As can be seen in Fig. 4A and B, the rate of the autophosphorylation reaction increased hyperbolically with time (i.e. it concaved down) whereas the rate of phosphorylation of the fusion protein increased parabolically with time (i.e. it concaved up) over the entire range of Fp(SH2) concentrations employed. In contrast to these results, the phosphorylation of Fp(SH2) by EGFR-P and EGFR-P autophosphorylation yielded families of straight line curves over the entire range of Fp(SH2) concentration employed

(Fig. 4C and D). Initial velocities of the Fp(SH2) phosphorylaytion reaction by EGFR (calculated from 15 s rates seen in Fig. 4A and B) are compared with initial velocities of the Fp(SH2) phosphorylaytion reaction by EGFR-P (calculated from the slopes of the curves shown in Fig. 4C and D) in Fig. 4E. Both curves are biphasic and far higher rates of Fp(SH2) phosphorylaytion by EGFR-P than by EGFR are observed similarly to what is seen in Fig. 2C.

The results of the experiments described above are in support of the concept that receptor autophosphorylation is a prerequisite for the interactions between EGFR and substrates containing SH2-domains and their eventual phosphorylation by the receptor [5]. This is clearly born out by the precursor-product kinetic pattern seen in Fig. 4A and B. It is also clear from these data that the phosphorylation of the receptor's highly reactive tyrosine residues is involved in these interactions as suggested by others [5,11].

Fig. 5 shows the results of experiments in which the phosphorylation of the fusion protein by EGFR and the autophosphorylation of EGFR in the absence or the presence of EGF are compared with the phosphorylation of the fusion protein by native and by truncated EGFR present in different membrane preparations and the autophosphorylation of these receptors. For the sake of comparison, the ordinates in Fig. 5A–E are expressed as 'relative activity' which is the extent of phosphorylaion of Fp(SH2) or of the autophosphorylation sites on the receptor by EGFR kinase observed at each time interval relative to the extent of the corresponding autophosphorylation reaction observed in the absence of Fp(SH2) in the system. As can be seen: (a) in all cases Fp(SH2) causes a slight inhibition of the autophosphorylation of the various receptors and (b) the phosphorylation of the fusion protein increases sigmoidally with increasing Fp(SH2) concentrations to a maximum observed at 0.45 μM Fp(SH2), and then decreases at higher fusion protein concentrations, as noted in the previous experiments.

The present results have shown that with ATP as variable, the phosphorylation by EGFR of a physiological, high affinity substrate derived from PLC-γ_1 follows a Bi-Bi sequential rapid equilibrium mechanism [8,9]. The $K_{ATP(Fp(SH2)}$ values are in the range of 1.0–1.47 μM (Table 1) compared with $K_{ATP(EGFR)}$ values in the range of 0.35 μM for normal EGFR whether receptor autophosphorylation is monitored in presence or absence of Fp(SH2). With the Fp(SH2) substrate as variable, the reaction kinetics are quite different than those observed with synthetic substrates such as polyGlu$_6$Ala$_3$Tyr or Angiotensin II [8]). Whereas the interactions between EGFR and the synthetic substrates are non-cooperative leading to Michaelis–Menten kinetics [8], in the interactions between EGFR and the physiological substrate (Figs. 2–5) there is a marked degree of positive cooperativity at Fp(SH2) concentrations lower that 0.45 μM, as judged from the sigmoidicity of the velocity curves, and a strong effect of substrate inhibition noted at higher Fp(SH2) concentrations which are still within the physiological range [14]. This is true in the case of purified EGFR as well as in the case of membrane-bound EGFR. Thus, the interactions between EGFR and a physiological substrate with SH_2-containing domains seem to involve mechanisms which allow the substrate to act as an on–off switch in the subsequent substrate phosphorylation reaction.

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