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Fibroblast growth factor phosphorylation and receptors in rod outer segments

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Acidic and basic fibroblast growth factors (aFGF and bFGF) have been isolated and purified from rod outer segments (ROS). aFGF is tightly bound to ROS membranes and can be specifically released by ATP. We show that this mechanism is dependent on the phosphorylation of aFGF itself. Phorbol 12-myristate 13-acetate (PMA) enhances this phenomenon independently of rhodopsin phosphorylation. This demonstrates that aFGF release from ROS membranes is dependent on its phosphorylation by endogenous kinase C. In addition specific binding sites for exogenous FGFs have been identified on ROS and disc membranes. A single high affinity site with a K_d of 40 pM was present in intact ROS while an additional low affinity site with a K_d of 300 – 600 pM was present in leaky ROS or in disc membranes. Light or ATP modified neither these K_d nor the apparent number of sites. The presence of specific receptors for FGFs and the kinase C dependent release of endogenous membrane bound aFGF suggest an autocrine mechanism which may be involved in photoreceptor cell biology.

Key words: fibroblast growth factor/phosphorylation/kinase C/photoreceptor/receptor

Introduction

Fibroblast growth factors (FGFs) have been implicated in neuronal development and survival after their identification as potent mitogens for many mesenchymal or ectodermal cells. They are thus multifunctional (reviews in Gospodarowicz and Schweigerer, 1986; Sporn and Roberts, 1988). FGF receptors have been localized on various cell types which are stimulated to divide by these growth factors. A plasma membrane associated receptor with a molecular weight of 125 – 145 kd (Neufeld and Gospodarowicz, 1985; Moscatelli, 1986; Moenner *et al.*, 1986) has been identified as the high affinity binding site. In some cases a low affinity specific binding site has also been reported and seems to be associated with proteoheparan sulphate synthesized by the cells or deposited in the basement membrane (Vigny *et al.*, 1988). The secretion of endogenous aFGF and bFGF (if any) by the tissues or cells which synthesize them is not yet understood since there is no coding sequence for a signal peptide in the aFGF and bFGF genes (Abraham *et al.*, 1986; Burgess *et al.*, 1986; Alterio *et al.*, 1988).

Acidic FGF (aFGF) was recently reported tightly associated with rod outer segment (ROS) plasma membrane, and could be specifically released by ATP (Plouët *et al.*, 1988) seemingly involving a phosphorylation mechanism. In addition, preliminary data suggested the presence of specific binding sites for FGFs in ROS. Recently receptors for other growth factors, IGF as well as insulin were described in ROS (Waldbellig *et al.*, 1987a,b). In this report the role of phosphorylation in the mechanism of release of endogenous aFGF in ROS by light and ATP is investigated in ROS and also in disc membranes and compared with rhodopsin phosphorylation. It is also of interest to study FGF receptors and their subcellular localization on membranes of the intact ROS or/and isolated disc membranes, and to compare the affinity and number of sites as a function of illumination. Our data suggest that there are two classes of FGF binding site, one on ROS plasma membrane and one on disc membrane and that the mechanism of endogenous aFGF release is mediated by kinase C dependent phosphorylation of the growth factor.

Results

Phosphorylation and release of endogenous aFGF in ROS

Using [γ-^{32}P]ATP under conditions known to release a maximum of aFGF from the disc and ROS membrane (condition I) (see Materials and methods), phosphorylated aFGF could be isolated by chromatography on a heparin – Sepharose column and eluted at 1 M NaCl PBS pH 7.4, and analysed by SDS – PAGE autoradiography (Figure 1) and HPLC (Figure 2). The autoradiographic

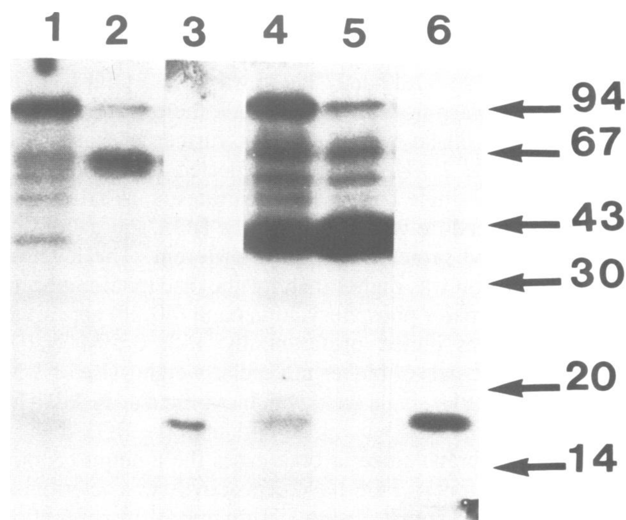

Fig. 1. Identification of [^{32}P]aFGF from disc membranes and ROS. Analysis of phosphorylated proteins extracted from disc membranes (**1 – 3**) and ROS (**4 – 6**) on SDS – PAGE gels. The total extracts (1 and 4) were loaded onto a heparin – Sepharose column (0.2 g). After an extensive wash with 0.65 M NaCl PBS (2 and 5) aFGF was eluted at 1.0 M NaCl PBS (3 and 6). In these experiments, the same amount of protein was treated and deposited on the gels.

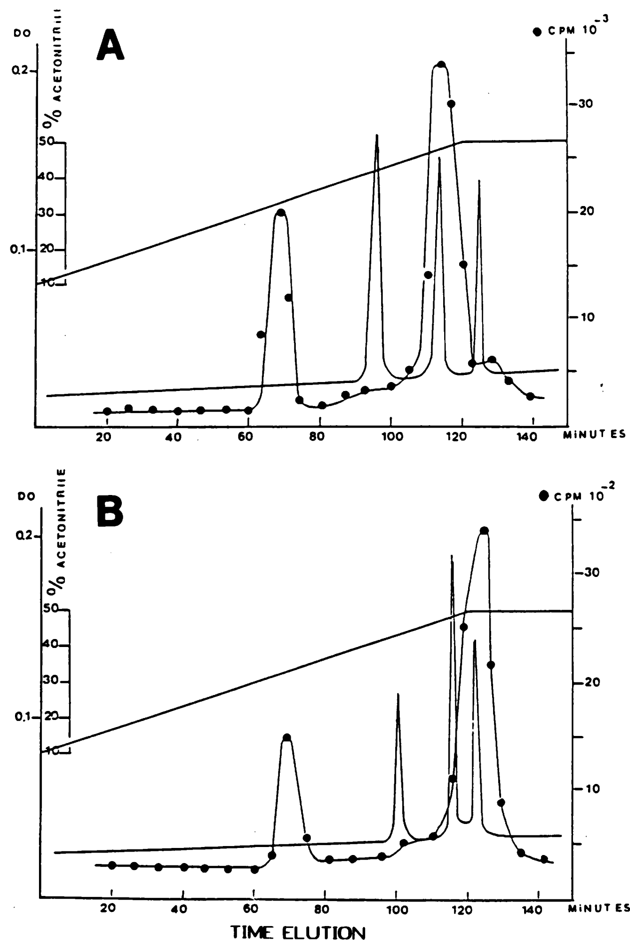

Fig. 2. Analysis of 1.0 M NaCl PBS elution of phosphorylated proteins on reverse phase HPLC. Phosphorylation in the presence (**A**) or absence (**B**) of PMA. Carrier bovine acidic FGF (gift from Choay Laboratory) was added to fraction 6 above and run as described previously (——) 280 nm absorbance, (●) ^{32}P. The last two peaks contained aFGF, determined by amino acid analysis.

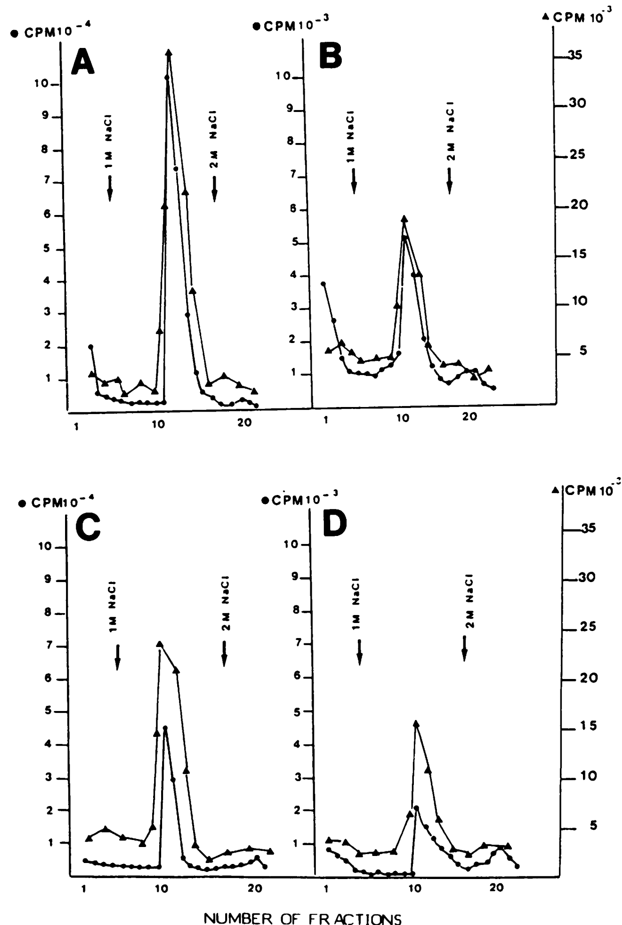

Fig. 3. Purification of phosphorylated aFGF released by PMA, light and ATP from ROS and determination of its biological activity. ROS were treated by two different methods. Condition I: no rhodopsin phosphorylation (**A** and **B**). Condition II: rhodopsin phosphorylation (**C** and **D**) in the presence (A and C) or in the absence (B and D) of 30 μM PMA. (●) ^{32}P; the biological activity was determined in BEL cells (Plouët *et al.*, 1984) (▲) by [^{3}H]thymidine incorporation.

pattern of the disc membrane showed six major bands, with different levels of phosphorylation, including a highly phosphorylated 95 kd band. There was also a faint band at 16 kd. After washing with 0.6 M NaCl, there was an elution of a band at 65 kd which was not further characterized (Figure 1, lane 2). However, as expected for aFGF, 1 M NaCl eluted a single band which migrates at 16 kd. When the same procedure was applied to the ROS, the pattern of phosphorylated proteins was quite different. The level of phosphorylation was higher than for the disc membrane and an additional major phosphorylation band (40 kd) appeared. This band was eluted preferentially with 0.6 M NaCl, together with bands of higher molecular weight. Use of 1 M NaCl also eluted a single band which migrated at 16 kd. The intensity of this band was much higher in the ROS than in the disc membrane since in both cases the amount of total membrane is similar. FGF biological activity was determined by its mitogenic activity using [^{3}H]thymidine incorporation into subcultured bovine epithelial lens (BEL) cells (Plouët *et al.*, 1984). The biological activity of phosphorylated aFGF was present in 1 M NaCl elution (see Figure 3B). The chromatographic pattern of the 1 M NaCl fraction on HPLC (Figure 2B) revealed that at least 75% of phosphorylated protein eluted at 1 M NaCl from heparin–Sepharose co-migrated with unlabelled aFGF at 45% acetonitrile. There was also a small phosphorylated peak at 32% acetonitrile. When rhodopsin was phosphorylated by light and ATP (condition II), aFGF could also be released from ROS but its biological activity and its phosphorylation level was lower than in condition I (Figure 3D).

Effect of phorbol ester (PMA) on aFGF phosphorylation and release

To investigate if endogenous kinase C was involved in this phenomenon, ROS prepared as in condition I were treated with PMA and the soluble proteins were run through a heparin–Sepharose column. HPLC profiles of 1 M NaCl eluant from heparin–Sepharose columns were similar in the presence (Figure 2A) or absence (Figure 2B) of PMA. PMA treatment greatly enhanced (20-fold) the aFGF phosphorylation (Figure 3A and B) level but there was no rhodopsin phosphorylation either with or without PMA under condition I (results not shown). The biological activity observed in the 1 M NaCl eluant showed that PMA released twice as much activity than without PMA (Figure 3A and B).

In order to investigate if rhodopsin kinase was also involved in aFGF phosphorylation, ROS membranes were processed under conditions permitting rhodopsin phosphorylation (condition II, see Materials and methods) in the presence or absence of PMA. In this case the biological

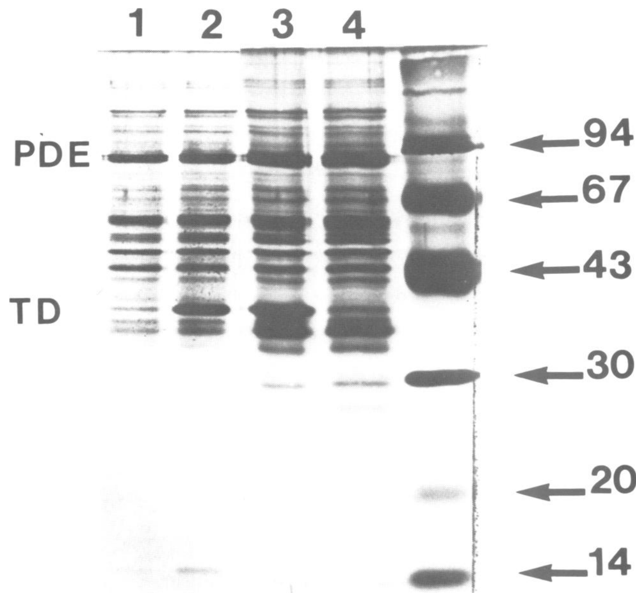

Fig. 4. SDS–PAGE of disc membrane (**1 and 2**) and ROS (**3 and 4**) soluble protein; membranes incubated with light (1 and 4) or in the dark (2 and 3). The ROS soluble proteins were stained by Coomassie blue and the disc soluble proteins by silver.

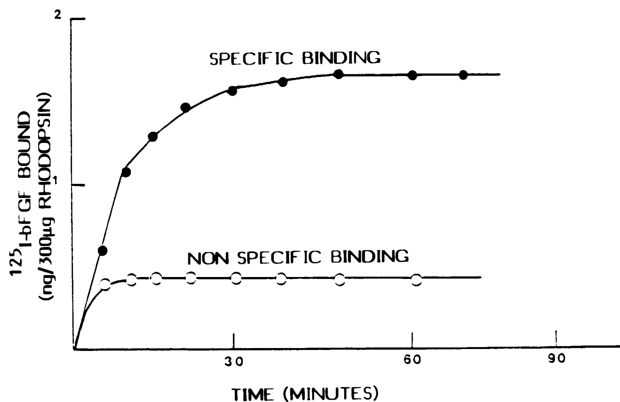

Fig. 5. Kinetics of [^{125}I]bFGF binding on ROS membranes: [^{125}I]bFGF was incubated with membranes at 4°C for various periods of time. Bound [^{125}I]bFGF was quantified after solubilization in 1% Triton X-100 by counting. Non-specific binding was measured in the presence of an excess of unlabelled factor (200-fold). Points are mean of triplicate samples.

activity of the 1 M NaCl eluant was enhanced (2-fold) by PMA (Figure 3C and D) as was its phosphorylation level (20-fold). However, both the activity and the level of aFGF phosphorylation remained 2-fold lower than in condition I. The results strongly suggested that there was a correlation between the release of aFGF mitogenic activity and the amount of phosphorylated aFGF. Rhodopsin phosphorylation was not required and did not prevent these phenomena.

Binding of a and b[^{125}I]FGF to different preparations of ROS membranes containing rhodopsin (R), opsin (R) and phosphorylated rhodopsin (P-R*)*

In order to determine the presence of FGF receptors in photoreceptors as well as their potential regulation as a function of rhodopsin activation, ROS membranes were treated by three different techniques and obtained in different conditions.

Three different treatments were employed: (i) dark adapted ROS (R); (ii) photoexcited rhodopsin (R*) and (iii) phosphorylated rhodopsin (P-R*). Two different methods were used to purify ROS: the first one according to Papermaster and Dreyer, (1974) (method A, see Materials and methods) led to the isolation of predominantly leaky ROS consisting of a disrupted plasma membrane surrounding stacks of discs. The second one, according to Uhl *et al.* (1987) (method B, see Materials and methods) is considered to yield predominantly intact ROS. To assess the role of ROS plasma membrane in the following experiments, we have also enriched membrane preparations for the disc in accordance with Puckett *et al.* (1985).

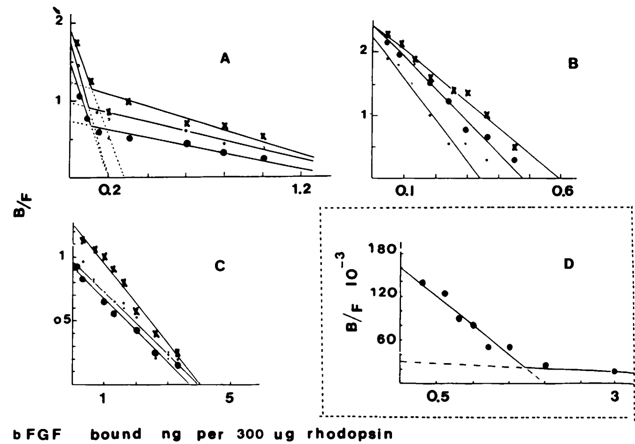

Fig. 6. Scatchard analysis of the binding of [^{125}I]bFGF to leaky ROS (**A**), intact ROS (**B**), disc (**C**) and BEL cells plasma membrane (**D**) prepared as described in Materials and methods. In each case, three different treatments of these membranes were used: dark adapted ROS (R) (●), opsin enriched ROS (R*) (○), phosphorylated rhodopsin (P-R*) (+). Scatchard analysis of the binding of [^{125}I]bFGF to BEL cells. [^{125}I]bFG bound was expressed in fmol/10^5 cells.

The Coommassie Blue/silver stained PAGE profile of the ROS and disc preparations is shown in Figure 4. Subsequent to extensive washing in isotonic buffer, hypotonic buffer washes of the disc (Figure 4, lanes 1 and 2) and ROS membranes (Figure 4, lanes 3 and 4) analysed by SDS–PAGE displayed the typical pattern of 'soluble and peripheral protein' as described by Kuhn (1981). One of these 'peripheral' proteins, transducin (TD), was tightly bound to membranes when the preparations were previously illuminated but was released in the dark membrane preparations. Phosphodiesterase (PDE) solubilization was independent of the light incubation conditions (Figure 4, lanes 1, 4 and 2, 3). None of these fractions could display bands of aFGF or bFGF due to the very low amount of these growth factors. We have also purified plasma membrane from bovine epithelial lens (BEL) cells. These membranes were used in the binding assay as positive controls to demonstrate the presence of specific receptors in the different ROS membrane preparations. The binding of [^{125}I]FGF to the ROS membrane preparations as a function of [^{125}I]FGF concentration reached an apparent saturation at room temperature in 45 min (Figure 5). Scatchard analysis of the binding showed two classes of binding sites for the leaky ROS prepared according to Papermaster and Dreyer (1974): one with a high affinity and one with a lower affinity, irrespective of the state of the visual pigment, i.e. dark (R), light (R*) or light ATP (P-R*) conditions (Figure 6A). With intact ROS only one high affinity site could be determined (Figure 6B). Conversely with enriched disc membranes on-

Table I. Comparison of the dissociation constants K_d and of the number of bound [^{125}I]bFGF per 300 μg of proteins in the different membranes preparations: R, R* and P-R*

Preparation	K_d (pM)		FGF bound (ng/300 μg)	
ROS				
A R	38	UD	0.34	UD
R*	39	UD	0.60	UD
P-R*	40	UD	0.48	UD
B R	35	550	0.32	1.50
R*	40	200	0.38	0.70
P-R*	42	450	0.38	1.30
C R	20	200	0.27	0.65
R*	25	150	0.30	0.75
P-R*				
D R	25	350	0.20	1.20
R*	27	225	0.28	1.29
P-R*	20	300	0.20	1.40
E R	300	2000	0.36	10.0
R*	350	3500	0.40	11.0
P-R*	295	3000	0.32	12.5
F R	UD	870	UD	3.70
R*	UD	750	UD	4.05
P-R*	UD	910	UD	3.95
G R	UD	735	UD	3.33
R*	UD	866	UD	3.24
P-R*				
BEL cells				
plasma membrane	150		2.0	
cells in culture	22	1200	12 000	100 0000[a]

(A) Intact ROS preparation according to Uhl *et al.*, 1987 (method B).
(B) ROS preparation as (A) but after one cycle of freezing and thawing, leading to leaky ROS.
(C) ROS preparations according to Papermaster and Dreyer (1974) (method A). This preparation yields a mixture of intact and leaky ROS.
(D) as in (C) but after one cycle of freezing and thawing.
(E) crude ROS after differential centrifugation but before purification on discontinuous sucrose gradient.
(F) Disc membrane preparation according to Puckett *et al.* (1985).
(G) as in (C) but after one cycle of freezing and thawing.
(H) BEL cells plasma membrane.
(I) BEL cells in culture[a] the number of binding sites are determined per cell.
(UD) Undetected; in intact ROS and in disc membrane preparations only the first and second binding sites were detectable. In BEL cell membranes only the first binding site was detectable.

ly the low affinity binding site was observed (Figure 6C). As with the leaky ROS, two classes of binding site were found with BEL cells in culture (Figure 6D). Table I shows the K_d and the number of sites per 300 μg of visual pigment under the different conditions for each membrane preparation.

For each membrane preparation, the K_d values and numbers of sites as a function of different rhodopsin treatments are within the limits of experimental variations and thus are not significantly different. As a positive control of FGF binding, plasma membrane preparations from BEL cells presented one high affinity binding site (Table I, H).

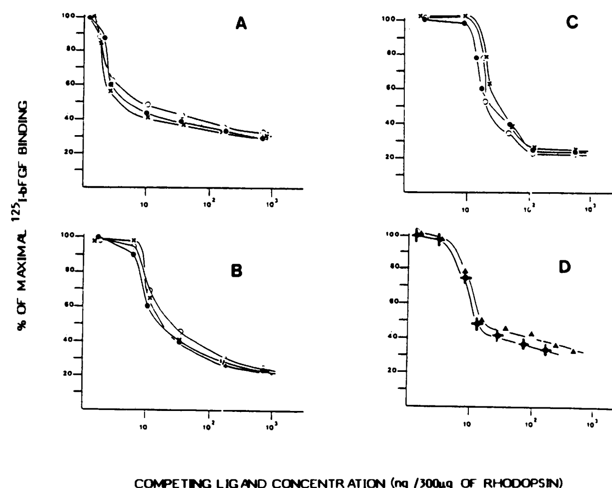

Fig. 7. Displacement of membrane-associated [^{125}I]bFGF by increasing concentrations of unlabelled bFGF and aFGF. Intact ROS (**A**), leaky ROS (**B**) and disc membranes (**C**) were incubated with [^{125}I]bFGF and different amounts of unlabelled bFGF were added as described in Materials and methods. In each case, different preparations were used as in Figure 3: R (●), R* (○), P−R* (+). Leaky ROS (**D**) were incubated with [^{125}I]bFGF and different amount of unlabelled bFGF (+) or aFGF (▲) were added.

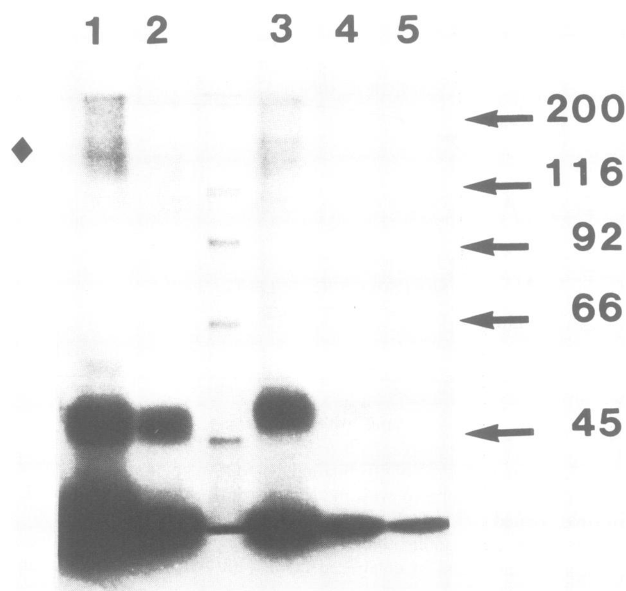

Fig. 8. Affinity labelling of [^{125}I]aFGF (**1** and **z**) and z[^{125}I]bFGF (**3–5**) with ROS prepared as described by Papermaster and Dreyer, 1974 (leaky ROS). The membranes were incubated as described in Materials and methods in the absence (1 and 3) or in the presence of 100-fold aFGF (2) or bFGF (4) or 500-fold bFGF (5). The upper bands (◆) correspond to one high mol. wt (~160 kd) affinity binding site. The second band (51 kd for aFGF and 53 kd for bFGF) corresponds to the second affinity binding site and migrates at the expected position for a rhodopsin−FGF heteromer.

Specificity of [^{125}I]bFGF binding

Competition experiments on the specific binding of [^{125}I]bFGF were performed by adding an increasing concentration of unlabelled FGF(s) to membrane preparations pre-incubated with labelled bFGF (Figure 7).

Incubation of intact (Figure 7A) or disrupted (Figure 7B) ROS membranes or disc membranes (Figure 7C) with a

constant amount of labelled bFGF and increasing amount of unlabelled bFGF (Figure 7A, B and C) and incubation of leaky ROS with aFGF (Figure 7D) resulted in competition of labelled FGF binding independently of the different treatments of rhodopsin. One half of the displacement was obtained with a 5- to 7-fold excess of unlabelled aFGF or bFGF in all competition experiments. The maximal displacement (70–80% of the total binding) for all the preparations was reached with a 200-fold excess of unlabelled FGF.

In leaky ROS the concentration of native basic FGF (50 ng) required to displace 50% of bound basic ^{125}I-labelled FGF (15 ng) was close to that for aFGF (Figure 7D), supporting the hypothesis of an identical site for both growth factors. There were no significant differences between the three treatments (R, R* and P-R*) in agreement with the binding assays analysed above.

Affinity cross-linking of aFGF and bFGF to ROS membrane

The nature of these receptors was further investigated by the affinity cross-linking of FGF to its binding sites.

When a leaky ROS preparation was incubated with ^{125}I-labelled a or bFGF in the presence of the homobifunctional cross-linker disuccinimidyl suberate (DSS) at 0.15 mM, the autoradiogram of the SDS–PAGE of these fractions revealed two major bands for the aFGF receptor complex (Figure 8, lanes 1 and 2) and two major bands and a minor one for the bFGF receptor complex (Figure 8, lines 3–5). The major bands for the aFGF receptor complex had apparent molecular weights of 160 and 51 kd. The major bands for bFGF receptor complex had apparent molecular weights of 160 and 53 kd; the minor band had an apparent molecular weight of 55 kd. The 160 and 55 kd bands disappeared when experiments were performed with an excess of aFGF (100-fold) or bFGF (100- to 500-fold). The 51 kd band for the aFGF receptor complex and the 53 kd band for the bFGF receptor complex were not completely displaced by a 100-fold excess of FGF. These results were consistent with the presence of two types of receptors on ROS: one on ROS plasma membranes and one on disc membranes. Acidic and basic FGF seemed to bind to the same receptors, with similar affinities. There was no significant influence of light and ATP on the binding of exogenous aFGF or bFGF to their specific receptors on ROS and disc membranes while we have previously found that these two effectors have a profound effect in the specific release of kinase C dependent phosphorylated endogenous aFGF.

The band at the bottom of the gel (M_r 13 kd) co-migrates with [^{125}I]FGF represents a ligand that was bound but not covalently attached; its apparent saturability is due to the removal of unbound FGF before the FGF cross-linking reaction. The M_r 160 kd and the M_r 55 or 53 kd undoubtedly represent the two kinds of affinity cross-linked FGF receptors [see the competition of unlabelled FGF with [~^{125}I]FGF on its receptors (Figure 8, lanes 2 and 4–5)].

Discussion

The presence of two different FGF receptors in ROS

The results reported in this work demonstrate the presence of FGF receptors on adult bovine photoreceptors. By the different techniques used, two types of receptors could be distinguished. The first one with the highest affinity (K_d 20–50 pM) seems to be localized on ROS plasma membranes since it could be determined in ROS and not on enriched disc preparations. The second one with the lowest affinity (K_d 300–600 pM) appears when leaky ROS were used or with disc preparations and thus is probably present only on discs. The number of binding sites is also different for the two receptors. The K_d of the high affinity site is similar to the values found for FGF receptors in other cells types in culture (Neufeld and Gospodarowicz, 1985; Moscatelli, 1986; Moenner *et al.*, 1986). In addition, the cross-linking experiments of [^{125}I]aFGF or [^{125}I]bFGF revealed an FGF receptor of high molecular weight (120–140kd) similar to the one described for other cell types. The K_d for the disc receptor does not seem to correspond to the value described for other cell types either to a low FGF affinity site associated with extracellular proteoglycans (K_d 2 nM, Moscatelli, 1986) or purified heparan sulphate proteoglycan (K_d 20 nM, Vigny *et al.*, 1988). It is tempting to attribute the disc FGF receptor to the 34 kd band observed by cross-linking experiments. In these conditions this band is co-localized with rhodopsin.

Whether this second receptor is rhodopsin itself or a minor protein which migrates at the same position on SDS–PAGE remains to be determined. Since rhodopsin represents >98% of integral disc membrane proteins, the low affinity binding may also reflect a degree of non-specific interaction.

In cell cultures, FGF receptors have been shown to be phosphorylated by FGF. This phosphorylation is mediated by a tyrosine kinase (Huang and Huang, 1986). In this report we also studied the role of ATP and light on FGF affinity to its receptors in photoreceptors. These two effectors trigger the phosphorylation of rhodopsin mediated by a rhodopsin kinase. In the radioreceptor assay, the data obtained with the different membrane preparations (ROS and disc membrane) do not show any significant effect of ATP or light on the affinity (K_d) or the number of sites of both receptors. The same result is obtained in competition assay. Thus the binding and the release of exogenous FGF is not ATP or light dependent. In these experiments bFGF is more efficient than aFGF in displacing [^{125}I]bFGF from the low affinity receptor. Transducin, a G-binding protein which interacts with rhodopsin in a GTP and light dependent manner does not displace FGF from these receptors, which is coherent with our previous data (Plouët *et al.*, 1988) on the lack of effect of GTP on aFGF release. The presence of FGF receptors on photoreceptor cells is not so surprising since their presence was demonstrated directly on neurons such as PC12 cells (Neufeld *et al.*, 1987) or indirectly by the neurotrophic effects of FGF (Walicke *et al.*, 1986). Other growth factor receptors for IGF and insulin (Waldbillig *et al.*, 1987a,b) have been found recently in ROS.

aFGF release is correlated with its own phosphorylation

In our previous work (Plouët *et al.*, 1988) we have described that photoreceptors, in particular ROS, contained aFGF and bFGF, and that endogenous aFGF–photoreceptor interaction involved a phosphorylation step. We have shown that only one third of the total amount of FGF contained in ROS can be solubilized by a hypotonic buffer and that

the remaining two thirds are tightly bound to ROS membranes. Only ATP can release endogenous aFGF from ROS and not GTP. In the present work, despite the fact that FGF's receptor might be phosphorylated *in vitro* by the binding of FGF by analogy with its behaviour in other cell types (Huang and Huang, 1988), ATP has no effect on the binding or on the release of exogenous [^{125}I]aFGF or [^{125}I]bFGF in the ROS or disc. Rhodopsin phosphorylation does not modify the amount of endogenous aFGF released. Thus it was of interest to examine whether the growth factor by itself and not the receptor could be phosphorylated. The data reported here clearly demonstrate that in the ROS and disc, endogenous aFGF released from membranes by ATP is phosphorylated. The presence of endogenous aFGF and its release by a phosphorylation mechanism do not involve the presence of specific receptor like the two types of receptors described in this work.

These data raise a new hypothesis of FGF anchorage in membranes, which may be a step in its secretion. This phenomenon remains still unexplained since aFGF cDNA does not code for a signal peptide sequence and has no strong hydrophobic region in its amino acid sequence (Abraham *et al.*, 1986; Jaye *et al.*, 1986; Gimenez-Gallego *et al.*, 1986; Alterio *et al.*, 1988).

In the presence of light in isotonic conditions, ATP phosphorylates rhodopsin and aFGF which allows a release of aFGF. In the absence of light, ATP phosphorylates and releases aFGF but does not phosphorylate rhodopsin. Thus aFGF and rhodopsin are not phosphorylated by the same mechanism and it can be concluded that the activation of rhodopsin kinase and rhodopsin phosphorylation are not directly involved in aFGF release and phosphorylation.

The presence of kinase C in ROS and its *in vivo* and *in vitro* activity (Kapoor *et al.*, 1987) as well as a recent report showing that kinase C can phosphorylate bFGF *in vitro* (Feige and Baird, 1988) suggested that phorbol esters may induce FGF phosphorylation. It is demonstrated here that PMA, a potent activator of this enzyme can stimulate aFGF phosphorylation in the ROS and disc, as well as its release. We show also that the amount of aFGF released from ROS and disc membranes is proportional to the aFGF phosphorylated. The phosphorylation sites on aFGF have not yet been determined in these conditions but our data show that the phosphorylation of aFGF does not modify its affinity to heparin–Sepharose. However, using mitogenic activity to test the biological activity of aFGF, we cannot exclude that aFGF biological activity might be significantly modified by its phosphorylation. The mechanism of FGF mobilization into the ROS, from disc membranes, independently of its interaction with a specific receptor and involving its phosphorylation may represent a new function for endogenous FGF in the cell. Endogenous FGF may act though a different way than exogenous FGF: after binding to its specific receptor, exogenous FGF enters into the cell by an endocytosis mechanism (Moscatelli, 1988; Bikfalvi *et al.*, 1988), and is then degraded, but it could also bind to the nucleus and activate ribosomal genes (Bouche *et al.*, 1988).

Recently, it was reported that when PMA is injected in rat eyes it does not induce rhodopsin phosphorylation *in vivo* while it does enhance the phosphorylation of several proteins, including a minor one at 15 kd (Kappor *et al.*, 1987). It will be of interest to examine whether this 15 kd protein is similar to aFGF.

Pleiotropic role of FGF in the retina

The results reported in this work demonstrate the presence of FGF receptors on adult bovine ROS and discs, extending our previous findings (Plouët *et al.*, 1988). We have already reported (Jeanny *et al.*, 1987) that embryonic as well as adult retina contain several FGF binding sites by incubating frozen eye sections with [^{125}I]FGF. One is localized on the basement membranes which surround the neural retina (inner limiting membrane and Bruch's membrane). This low affinity (K_d 20 nM) binding is associated with heparan sulphate proteoglycan localized in these basement membanes. The other sites are localized in the neuronal layers within the retina. Their nature has not yet been fully determined (Halley *et al.*, 1988) but their relative insensitivity to heparitinase treatment makes them likely to be similar to the high affinity site observed in cultured cells or in purified membrane preparations with a K_d of 200 pM. In this study, however, ROS does not seem to be the subcellular part with the largest number of FGF receptors in adult bovine retina.

We cannot exclude the possibility that in the ROS the low affinity site is due to the presence of heparan sulphate proteoglycan of different composition than the one which can be visualized by the antibody against mouse EHS extracted heparan sulphate proteoglycan (Vigny *et al.*, 1988). This antibody revealed the presence of heparan sulphate proteoglycan in the basement membrane but not in the neural retina (Halloui *et al.*,1988). Recent studies with *in vitro* culture of chick photoreceptor cells have demonstrated that heparan sulphate proteoglycan can be synthesized by these cells (Needham *et al.*, 1988). However, this hypothesis is unlikely to be due to the relative insensitivity of FGF-binding sites within the neural retina to digestion by heparitinase or heparitinase in eye sections.

In conclusion, aFGF is an intrinsic component of photoreceptor cells, as shown by this work and by immunocytochemical localization. It is also synthesized by these cells, as we have shown recently by *in situ* hybridization of an aFGF cDNA cloned from bovine retina mRNA (Halley *et al.*, 1988) on eye sections. In addition ROS contain specific receptors for FGF, as demonstrated in the present work. We have also shown recently that aFGF enhances rhodopsin synthesis in newborn rat photoreceptor cells *in vitro* (Hicks and Courtois, 1988). All these findings seem to indicate that aFGF may have a pleiotropic role in photoreceptor physiology and that phosphorylation of the growth factor is an important step in its mechanism of action in ROS. Whether or not FGF is involved in phototransduction will require further study, showing for instance that it may be involved in light dependent Ca^{2+} movement or interacts with proteins involved in the visual cascade and regulates phototransduction, as for the S antigen (Zucherman and Cheasty, 1986). This may represent a useful model for studying the mechanism of action of FGF release in parallel with its mitogenic activity in cell culture.

Materials and methods

ROS preparation

The first ROS purification protocol used (method A) followed with slight modifications the methods using stepwise gradients of sucrose (Papermaster

and Dreyer, 1974) and further continuous gradients (De Grip *et al.*, 1980).

Briefly, bovine eyes were collected soon after death at the local slaughter house and kept in the dark for at least 3 h so as to be fully dark-adapted. The retina was removed carefully under dim red light and resuspended in a sucrose homogenizing medium containing 45% sucrose (w/w), 70 mM sodium potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA. All subsequent steps were performed in the dark. The ROS were floated on 45% sucrose and then sedimented in 15% sucrose. After homogenization, membranes were layered on top of a discontinuous gradient buffer containing sucrose in incremental density steps of 1.11, 1.13 and 1.15 g/ml with 70 mM sodium potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA (Buffer A).

Purified ROS were collected at the 1.11–1.13 g/ml sucrose interface. After diluting with buffer A, washing and pelleting, the purified ROS were stored in the dark at −70°C. The procedure yields predominantly 'leaky' ROS, i.e. ROS fragments with a perforated plasma membrane. ROS obtained by this method will be referred to as leaky ROS.

To facilitate rapid isolation and purification of intact rod outer segments a second method was used (Uhl *et al.*, 1987) (method B): the retina were dissected under dim red light and placed in a modified Ringers' solution pH 7.3 (120 mM NaCl, 4 mM KCl, 10 mM glucose, 10 mM Hepes, 2 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA. After vortexing the retina, the ROS were filtered through a Teflon screen (125 mesh). The filtered solution was layered on a stepwise gradient of sucrose (1.14–1.15 g/ml).

After centrifugation the intact ROS were collected at the upper interface and washed in Ringers' solution, pelleted and stored in the dark at −70°C or used for further experiments.

Disc membrane preparation

This preparation used the procedure previously described by Puckett *et al.* (1985) without the 5–20% Ficoll 400 density gradient.

Briefly, fresh retinas were homogenized in 45% sucrose (w/w) in 100 mM potassium phosphate (pH 7.0) and 5 mM mercaptoethanol (Buffer B), and centrifuged. Then the supernatant was washed in buffer B without sucrose. The ROS were pelleted by centrifugation, resuspended in 40% sucrose (w/w) in buffer B, and centrifuged. The ROS were further diluted 8-fold with 5 mM β-mercaptoethanol, centrifuged and the pellet resuspended in 5% Ficoll 400 (w/w) and 5 mM β-mercaptoethanol. After 4 h, the ROS were osmotically lysed and sedimented; osmotically intact discs were collected from the surface of the 5% Ficoll 400, washed extensively with 5% sucrose (w/w) in buffer B and stored in the dark at −70°C or used for further experiments.

Specific release of FGF and ROS and disc membrane by light, ATP and PMA

In one set of experiments, ROS or enriched disc preparations were treated as previously described (Plouët *et al.*, 1988) (condition I): membranes were bleached for 10 min at 20°C with white light (150 W) at 20 cm in a hypotonic buffer containing 5 mM Hepes (pH 7.4), 1 mM $MgCl_2$ and 2 mM DTT, centrifuged and the membrane pellet treated with 3 mM ATP in hypotonic buffer in the dark for 1 h with or without 30 μM PMA to obtain full dissociation of FGF mitogenic activity from the membrane.

In a second series, membranes from ROS or enriched disc preparations were treated to obtain phosphorylated rhodopsin (Mc Dowell and Kuhn, 1977) in the presence or absence of 30 μM PM (condition II).

The membranes treated with the two different protocols were centrifuged in a Beckman TL 100 ultracentrifuge at maximum speed for 10 min at 4°C. After addition of an excess of 0.65 M NaCl PBS the supernatants were applied to a heparin–Sepharose column equilibrated with 0.65 M NaCl PBS (pH 7.4) and extensively washed. Samples from the 1 M NaCl PBS and 2M NaCl PBS elutions were analysed for their FGF mitogenic activity with the FGF bioassay. The ^{32}P-phosphorylated proteins retained on heparin–Sepharose and eluted at 1 M–2 M NaCl PBS were also analysed by autoradiography on SDS–PAGE or analysed on HPLC (Mascarelli *et al.*, 1987).

Treatment of the different ROS membrane preparations

Three different preparations of ROS membranes were used to study [^{125}I]FGF binding to its receptor.

The first was a dark adapted ROS preparation which was kept in the dark during all the steps from the isolation until the binding assay. It will be referred to as the 'rhodopsin preparation' (R).

The second preparation was opsin enriched ROS, prepared by bleaching the ROS suspension with white light (150 W) at a distance of 20 cm, for 30 min at 30°C. The tube containing the ROS were slowly rotated during the illumination. This bleached ROS preparation was then kept in the dark for at least 4 h at 30°C. It will be referred to as the 'opsin preparation' (R*).

The third ROS membrane preparation was incubated in the dark with a phosphorylating buffer containing 3 mM ATP, 10 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.3). After mild sonication in a water bath at 30°C and a few minutes to allow temperature equilibration, the ROS membranes were bleached with white light (150 W) for 2 h to phosphorylate maximally rhodopsin (Kuhn, 1981). It will be referred to as the 'phosphorylated rhodopsin preparation' (P-R*).

The binding assay was performed in Eagle's minimum essential medium (MEM) containing 1 mg/ml of bovine serum albumin (BSA).

Purification of the plasma membrane from BEL cells

BEL cells were grown to confluency in MEM containing 6% foetal calf serum (FCS), glutamine 1%, penicillin 100 U/ml and tetramycine 100 ug/ml (Eurobio) in 14-cm Petri dishes (Arruti and Courtois, 1978). Each dish was washed twice with 15 ml cold PBS, then 5 ml PBS was added and the cells were scraped off with a Rubber policeman. After a first centrifugation, the pellet was resuspended in $ZnCl_2$ 1 mM in bidistilled water for 10–15 min, centrifuged again for 15 min and the resulting pellet resuspended in bidistilled water (these two steps were done at room temperature). After 10 min, the cells were centrifuged for 15 min and the pellet resuspended in 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 (pH 8.0) and 3 mM $MgCl_2$ (Buffer C) at 4°C.

The resulting cell suspension was homogenized at 4°C in a dounce type B homogenizer with 150 strokes. The homogenate was centrifuged for 15 min at 3500 r.p.m. (minifuge heraeus); the pellet was resuspended in 10 ml of the upper phase of a decanted solution containing dextran 500 and polyethylene glycol 6000 [200 g solution dextran 500 at 20% (w/w) in bidistilled water; polyethylene glycol 6000 at 30% (w/w) in bidistilled water; 333 ml from a 220 mM mono-disodique phosphate (pH 6.5) and 179 ml bidistilled water]. After a second centrifugation in the minifuge for 5 min at 4000 r.p.m. the resulting pellet was mixed with an equal volume of the upper and lower phases of the dextran–polyethylene glycol, centrifuged for 10 min at 2000 r.p.m. without breaking and the supernatant was vigorously shaken and centrifuged again for 15 min at 2500 r.p.m.

The pellet was resuspended with the two phases in equal volumes, shaken and centrifuged for 20 minutes at 3000 r.p.m. The interface was collected, diluted twice with the two phases, shaken and centrifuged for 15 min at 3500 r.p.m. This last step was repeated until the pellet had completely disappeared.

The final collecting interface was centrifuged for 30 min at 4000 r.p.m. and the membranes were stored in 20% glycerol, 1‰ azide and frozen at −20°C.

Cell culture and FGF bioassay

Monolayers of BEL cells were cultured in Eagle's MEM containing 6% FCS in 24- well dishes (Falcon) (Arruti and Courtois, 1978). FGF bioassay was performed using [^{3}H]thymidine incorporation during the last 4 h of the growth factor stimulation period in subcultured BEL cells between the 11th and the 20th passage (Plouët *et al.*, 1984).

Results are expressed as the amount of protein providing 50% of the maximal stimulation (stimulation unit, SU) in comparison to standard curves obtained with purified FGF. Using this method, the measured mitogenic activity was very reproducible because it was determined on the linear portion of the S-shaped curve from dose–response experiments. For each sample a dose–response curve of activity was performed in triplicate.

SDS–PAGE and protein determination

The soluble proteins from the different ROS and disc membranes, the proteins purified on the heparin Sepharose columns and FGF cross-linked to its receptor were analysed by SDS–PAGE (Laemmli, 1970). The gels were stained with Coomassie brillant blue or silver (Wray *et al.*, 1981); the protein concentration was measured using BSA as standard (Bradford, 1976).

Growth factor purification

Purified aFGF and bFGF were isolated from bovine brain using a heparin–Sepharose affinity technique (Courty *et al.*, 1985). They were further purified by HPLC and their purity controlled by amino acid determination.

Purification of the G binding protein (transducin) contained in the ROS preparation

Transducin was purified according to Kuhn *et al.* (1981). ROS purified as described above were washed extensively in the dark with an isotonic buffer:

70 mM sodium-potassium phosphate (pH 7.4) 1 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA, and centrifuged for 20 min at 50 000 *g* at 4°C. The supernatant containing the soluble protein was discarded and the pellet was bleached with a white light (150 W) at 20 cm for 15 min in the presence of a hypotonic buffer 5 mM Hepes (pH 7.0), 1 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA to elute phosphodiesterase (PDE). After a second centrifugation, the resulting pellet was washed again with a hypotonic buffer (5 mM Hepes (pH 7.0), 2 mM DTT and 0.5 mM GTP); the supernatant contained the transducin. To ensure purification to homogenecity and to discount eventual contamination we used a mono-S chromatography column (Pharmacia).

aFGF and bFGF radioreceptor assay

Purified bovine aFGF and bFGF were labelled with ^{125}I according to the chloramine T method (Hunter and Greenwood, 1962).

FGF (2–4 μg) in 20 μl 10 mM Tris–HCl (pH 7.4) 0.5 M NaCl was added to 0.5–1 mCi of Na[^{125}I] (CEA, France) and 5 μl of chloramine T at 1 mg/ml for 15–20 s. Then to arrest the iodination reaction 5 μl $Na_2S_2O_5$ at 2 mg/ml was added. Five minutes later, 10 μl of NaI were added and the mixture was applied to a 1 M heparin–Sepharose column (0.1 g) equilibrated with 0.65 M NaCl PBS (pH 7.4). After thoroughly washing with this buffer, [^{125}I]aFGF or [^{125}I]FGF were eluted with 1 M NaCl PBS or 2 M NaCl PBS, respectively. [^{125}I]FGF was then desalted on a PD10 column (Pharmacia), aliquoted and stored at −20°C.

Rhodopsin (500 μg) for ROS and disc membranes or total protein (10 μg) for the BEL cell plasma membranes were used for each binding assay. The binding was performed in the dark by incubating in TL 100 Beckman ultracentrifuge tubes (in a final volume of 300–500 μl) the differently treated membranes with MEM (pH 7.4) containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml BSA (binding buffer). Iodinated FGF at the desired concentration was then added for various periods of time to determine the kinetics of specific ^{125}I-labelled FGF binding to the different membrane preparations. Non-specific binding of [^{125}I]FGF to these membranes was determined in the presence of an excess of unlabelled FGF (200-fold). During the binding assay the tubes were incubated at room temperature on an oscillating platform set at 3 cycles/s. (Bioblock).

Following incubation, 500 μl of cold binding buffer was added, the tubes were centrifuged for 10 min at 100 000 r.p.m. at 4°C, the supernatant was discarded and the pellet was washed four times more. Membrane associated radioactivity was determined by counting the pellets in a gamma counter (LKB 1275). The specific binding was then analysed by the Scatchard method (Scatchard 1949).

[^{125}I]bFGF competition assay

Competition experiments on the specific binding of [^{125}I]bFGF on three different membrane preparations were done with constant amounts of labelled bFGF: 2 ng per 300 μg of rhodopsin with intact ROS (method B), 4 ng per 300 μg of rhodopsin with leaky ROS (method A) and 10 ng per 300 μg of rhodopsin with the enriched disc membrane preparations. After incubation with [^{125}I]bFGF for 1 h, the membranes were incubated at room temperature on an oscillating platform in the serum free binding buffer with increasing amounts of unlabelled bFGF over a range of 2–1000 ng per assay. After 45 min the membranes were centrifuged, the medium removed and the different membrane pellets were washed four times with cold binding buffer. The membrane associated radioactivity was determined as described above.

Cross-linking of [^{125}I]FGF to their receptors on the different membrane preparations

ROS with plasma membrane attached to disc membranes (leaky ROS) and disc membranes were incubated for 1 h at room temperature with [^{125}I]FGF. Control samples received in addition a large excess of unlabelled FGF. Throughout the procedure, samples were shaken on an oscillating platform at 3 cycles/s. At the end of the incubation the membranes were centrifuged and washed with the binding buffer as above. The cross-linking reagent DSS (dissolved in dimethyl sulphoxide to a concentration of 15 mM and then diluted with PBS to a final concentration of 0.15 mM) was added to the different preparations (at room temperature) for 15 min. The cross-linking reaction was arrested by adding a solution of 10 mM Tris–HCl (pH 7.5), 200 mM glycine, 1 mM EDTA and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF) at a ratio of 1:100 in the cross-linking buffer. After a few minutes the membranes were washed with ice cold PBS, the pellet was resuspended in PBS containing 1% Triton X-100, 1 mM EDTA and 0.1 mM PMSF, and centrifuged. The supernatants were analysed by SDS–PAGE.

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Notes added

1. After this paper was submitted, it was reported that rhodopsin could be a receptor for bFGF while the value for its affinity (K_d 6 nM) differed from our data K_d (300–600 pM). Plouët,J. (1988) *J. Cell. Biol.*, Supplement 12B, Alan Liss, New York pp. 225.

2. Dr A.Baird has recently brought to our attention a forthcoming report (Feige,J.J. and Baird,A. (1989) *Proc. Natl. Acad. Sci., USA*, in press) where it is shown that a- and bFGF can be phosphorylated *in vitro* by different kinases. bFGF is also phosphorylated *in vivo* in endothelial vascular cells. These results tend also to show that FGF phosphorylation may be an important step for its mechanism of action.