Signaling Initiated by Overexpression of the Fibroblast Growth Factor Receptor-1 Investigated by Mass Spectrometry*

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Overexpression of the fibroblast growth factor receptor-1 (FGFR-1), a prototypic receptor tyrosine kinase, is a feature of several human tumors. In human 293 cells overexpression of the FGFR-1 leads to constitutive activation of the receptor with concomitant sustained high increase in the cellular level of phosphotyrosine-containing proteins. Here we use mass spectrometry to study the tyrosine-phosphorylated proteins induced by overexpression of the FGFR-1. Several well known components of FGFR-1 signaling were identified along with two novel candidates: NS-1-associated protein-1 and target of Myb 1-like protein. We subsequently applied mass spectrometry precursor ion scanning to identify 22 tyrosine phosphorylation sites distributed on six substrate proteins of the FGFR-1 or downstream tyrosine kinases. Novel in vivo tyrosine phosphorylation sites were found in the FGFR-1, phospholipase Cγ, p90 ribosomal S6 kinase, cortactin, and NS-1-associated protein-1 as a result of sustained FGFR-1 signaling, and we propose these as functional links to downstream molecular and cellular processes. Molecular & Cellular Proteomics 2:29–36, 2003.

Receptor tyrosine kinases convey extracellular stimuli to intracellular signaling. When activated they induce tyrosine phosphorylation of specific cytosolic substrates and thereby create highly specific binding sites for the Src homology 2 (SH2)1 or phosphotyrosine (Tyr(P)) binding domains in proteins that will further promote intracellular signaling cascades (1). The human genome encodes 58 different receptor tyrosine kinases (2), which are involved in events such as proliferation, differentiation, metabolic control, and migration.

Overexpression of receptor tyrosine kinases can lead to constitutive activation as first described for the ErbB2 receptor (3) and to concomitant elevated cellular tyrosine phosphorylation status. Overexpression and amplification of the fibroblast growth factor receptor-1 (FGFR-1), a prototypic receptor tyrosine kinase, have been found in tumor samples from human pancreas (4, 5), breast (6, 7), brain (8, 9), prostate (10), thyroid gland (11), and salivary gland (12). To understand the role of FGFR-1 in cancer it is vital to define the signaling pathways that confer the oncogenic potential and how they differ from the “healthy” situation.

In a recent study, immunoprecipitation with anti-Tyr(P) antibodies combined with SDS-PAGE and mass spectrometry was used to identify proteins that were tyrosine-phosphorylated as a result of epidermal growth factor stimulation of HeLa cells (13). Two novel candidates in the extensively studied epidermal growth factor receptor signaling pathway were isolated, and substrates present in femtomole amounts were identified. Techniques for tracing phosphopeptides in complex mixtures by MS have been developed and are now finding applications in phosphorylation studies of in vivo samples (14). These developments, however, have primarily been used in the characterization of serine and threonine phosphorylations due to (i) their relative abundance (>99.9%) compared with tyrosine phosphorylation (15) and (ii) the lability of the aliphatic phosphoester bond, which readily undergoes β-elimination. The latter has been a focus point in the development of MS-based techniques for phosphopeptide tracing. It does not, however, apply to the more stable aromatic phosphoester bond of phosphotyrosine residues.

Nanoelectrospray MS precursor ion scanning of peptide mixtures in the negative ion mode has previously been used for phosphopeptide analysis by means of monitoring the specific marker ion at m/z −79 (representing the PO2− fragment ion released from all phosphopeptides) (16, 17). Steen et al. (18) recently extended this work by developing a precursor ion scanning procedure in the positive ion mode, which specifically detects tyrosine-phosphorylated peptides. First, collision-induced fragmentation of precursor ions containing Tyr(P) residues leads to formation of the Tyr(P) immonium ion (im(Tyr(P))) that is detected as a reporter ion at m/z 216.043. Second, im(Tyr(P))-containing precursor ions are sequenced,
and the phosphosite is mapped by MS/MS.

We combined the strategy of Pandey et al. (13) and the development of Steen et al. (18) to elucidate the signaling cascade induced by overexpression of the FGFR-1 in human 293 cells. Proteins either tyrosine-phosphorylated, or complexed with these, were identified using anti-Tyr(P) immunoprecipitation, one-dimensional SDS-PAGE, and nondenaturing gel electrophoresis (SDS-PAGE). Subsequently we applied precursor ion scanning for im(Tyr(P)) to map tyrosine phosphorylation sites. Nine proteins were identified by MS to be specifically tyrosine-phosphorylated, or complexed with a tyrosine-phosphorylated protein, as a result of FGFR-1 overexpression. Two of these, NS-1-associated protein-1 (NSAP1) and target of Myb 1-like protein (TOM11), have not previously been described in FGFR signaling. A total of 22 in vivo tyrosine phosphorylation sites were mapped, including novel sites in phospholipase Cγ (PLCγ), p90 ribosomal S6 kinase (p90RSK), cortactin, NSAP1, and the FGFR-1 itself.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—293T (human epithelial kidney) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. For transfections, cells were harvested and resolved in full medium without antibiotics. Electroporation was performed at 310 V in a Cellject PRO (Hybaid). 24 h post-transfection antibiotics were added to the cell cultures.

**Immunoprecipitation, SDS-PAGE, and Western Blotting**—48 h post-transfection −2 × 10⁷ cells were lysed in 15 ml of lysis buffer (phosphate-buffered saline, pH 7.4 (Sigma), 1% Nonidet P-40, 1 mM sodium orthovanadate, and Complete protease inhibitors (Roche Diagnostics)). Cleared cell lysates were incubated overnight at 4 °C with a mix of 4G10 (70 µL, Upstate Biotechnology) and PY99 (30 µL, Santa Cruz Biotechnology) monoclonal anti-Tyr(P) antibodies covalently coupled to agarose beads. Precipitated immune complexes were washed twice in lysis buffer, washed twice in phosphate-buffered saline alone, and then eluted with 150 µL phenyl phosphate in phosphate-buffered saline. Elution fractions were precipitated with 12% trichloroacetic acid, washed in acetone, redissolved and boiled 5 min in 2× SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were visualized using colloidal Coomassie staining (Coomassie Brilliant Blue G-250, Bio-Rad).

For Western blotting, identical amounts of protein were resolved by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and immunoblotted with antibodies against Tyr(P) (4G10, Upstate Biotechnology) or doubly phosphorylated (Thr(P)-202/Tyr(P)-204) extracellular signal-regulated protein kinase 1 (Erk1)/Erk2 (Cell Signaling Technology) according to the manufacturer’s protocol.

**Mass Spectrometry**—Proteins were excised from the colloidal Coomassie-stained polyacrylamide gel and reduced, alkylated, and digested with trypsin as described previously (19). Tryptic peptide mixtures were desalted on Poros R2 sorbent (Perceptive Biosystems) in GELoader tips (Eppendorf) and eluted into nanoelectrospray needles (Protana Engineering, Odense, Denmark) as previously described by Neubauer and Mann (17). Mass spectrometry was performed on an API QSTAR Pulsar Hybrid quadrupole time-of-flight mass spectrometer (AB-MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (Protana Engineering, Odense, Denmark).

Precursor ion scanning was conducted as described previously (18) with a few modifications. Pulsed Q2-ion injection was optimized for transmission of the im(Tyr(P)) (the ion release pulse delay and ion release width between the IQ3 pulse and the Push/Pull pulses of the time-of-flight modulator were set at 50 and 27 µs, respectively). MS/MS spectra were acquired using collision-induced dissociation of selected peptide ions, generating characteristic b- and y-type fragment ions (20). Peptide sequence tags (24) of y- and/or b-ion series were searched against the National Center for Biotechnology Information (NCBI) non-redundant database (NRDB) using Pepsea (MDS Proteomics, Odense, Denmark). Furthermore, all MS/MS spectra from each gel band were centroided and merged to a single file, which was searched using a breakpoit algorithm against the mammalian NCBI NRDB via the Mascot Search Engine (www.matrix-science.com/).

**RESULTS**

**Tyrosine Phosphorylation and Erk Activation in Human 293 Cells Overexpressing Human FGFR-1**—Human 293 cells were transiently transfected with a vector encoding the full-length, three Ig domain, isoform of human FGFR-1 and harvested after 48 h. Western blotting with antibodies against Tyr(P) and activated Erk1/Erk2 revealed highly elevated cellular levels of Tyr(P)-containing proteins and Erk activation (Fig. 1). Erks are hallmark downstream signaling molecules in growth factor
receptor signaling (21–23), and we conclude that overexpression of the FGFR-1 leads to constitutive activation of the intrinsic tyrosine kinase with subsequent downstream signaling involving Erks. To further investigate the signal transduction we used immunopurification with anti-Tyr(P) antibodies to selectively purify proteins that are phosphorylated by the FGFR-1 or a downstream kinase or are bound in a complex with a protein that is tyrosine-phosphorylated upon FGFR-1 overexpression. We used saturating conditions during the immunoprecipitation and phenyl phosphate solution for elution to minimize contaminant protein in the elution fractions. Eluates from FGFR-1-overexpressing and control cells were compared using SDS-PAGE with colloidal Coomassie staining (Fig. 1). Gels from three experiments were compared, and visualized protein bands exclusive to, or of highly increased intensity in, the FGFR-1-transfected cells in all three experiments were excised for analysis by mass spectrometry.

Identification of Tyrosine-phosphorylated or Associated Proteins by Nanoelectrospray MS/MS—Peptides were selected for MS/MS from an initial MS analysis. MS/MS was performed by collision-induced dissociation of peptide ions in the second quadrupole of the quadrupole time-of-flight mass spectrometer, thus generating the characteristic b- and y-type fragment ions. The fragment ion spectra were analyzed by peptide sequence tag search using localized intrapeptide type fragment ions. The fragment ion spectra were analyzed by peptide sequence tag search using localized intrapeptide partial amino acid sequence information for identification of the peptide and ultimately the protein (24). Fig. 2 illustrates the identification of NSAP1 by MS/MS (protein band 8 and 9 in Fig. 1). Peptide ions (indicated by arrows in Fig. 2A, which shows a selected region of the MS spectrum) were chosen for MS/MS fragmentation. Fig. 2B shows the y-ion series generated by MS/MS fragmentation of the triply charged ion at m/z 491.7 (indicated by a bold arrow in Fig. 2A) to unequivocally identify the protein as NSAP1. In addition, a merged file of all MS/MS spectra from individual bands was searched using a breakpoint algorithm for complementary data validation.

Sequence coverage ranged between 8 and 34%; however, unambiguous protein identification and localization of phosphorylation sites rather than high sequence coverage per se was the aim of this study. Table I lists the nine proteins identified from first round nanoelectrospray MS/MS analysis. In addition, the protein sequences used to identify two new proteins in FGFR-1 signaling, NSAP1 and TOM111, are presented in Fig. 4A.

Identification of Tyrosine-phosphorylated Peptides by Precursor Ion Scanning—After the initial MS/MS for identification of the proteins, the mass spectrometer was converted to precursor ion scan mode to identify peptides releasing the im(Tyr(P)) observed at m/z 216.043. In short, the first quadrupole of the mass spectrometer was set to scan over a desired mass range; the second quadrupole was used as a collision cell with parameter settings allowing for internal peptide fragmentation. A peptide containing a phosphorylated tyrosine will release an immonium derivative of the phosphorylated tyrosine residue, im(Tyr(P)), which is specifically detected in the time-of-flight section.

Peptide ions found to release im(Tyr(P)) were analyzed by MS/MS to identify the site of phosphorylation. Fig. 3 shows the precursor ion scan of the tryptic digest containing PLCγ (protein band 1 in Fig. 1). The four MS/MS spectra identifying the four tyrosine phosphorylation sites are also shown. In all cases the sequence tag unambiguously located the phosphorylation site. In tryptic peptides with more than one tyrosine residue, the sequence information obtained from the MS/MS data would either cover the phosphorylated tyrosine residue (Fig. 3C, spectrum of PLCγ residues 764–778) or alternatively yield sufficient sequence coverage to discriminate between
two tyrosine residues (Fig. 3C, spectrum of PLCγ residues 466–491). Table I lists 22 tyrosine phosphorylation sites from six different proteins that were identified by precursor ion scanning, and in addition, the precursor ion scan and MS/MS spectrum identifying the phosphorylation site in the NSAP1 is presented in Fig. 4B. These phosphorylations all appear to be downstream of FGFR-1 kinase activity since immunoprecipitation with anti-Tyr(P) antibodies from cells transfected with a dominant negative (kinase-deleted) FGFR-1 mutant did not reveal any bands that were not also present in the immuno precipitate from control cells (data not shown).

Indicative of the efficiency of the method, all three of the well described in vivo tyrosine phosphorylation sites in PLCγ (25, 26) and six of seven well characterized phosphorylation sites in the FGFR-1 were identified (27). In addition a total of five novel in vivo tyrosine phosphorylation sites were identified in these two proteins. Phosphorylation of Tyr-766 in the FGFR-1 is necessary for recruitment of PLCγ/H9253 in these two proteins. Phosphorylation of Tyr-766 in the FGFR-1 were identified (27). This phosphorylation site was not identified, although PLCγ was clearly recruited to the receptor. A limitation of the method is the analytical mass range of the tandem mass spectrometer, which means that peptides above 3000–4000 Da are usually not detected with current instrumentation. Investigation of the protein sequence revealed that the tryptic peptide fragment containing Tyr-766 is greater than 3.3 kDa, thereby providing a reasonable explanation as to why the peptide was not observed. Also, the chemical properties of a given peptide (e.g. acidity and hydrophilicity) influence its ability to be detected by MS. Phosphorylated peptides are often both acidic and hydrophilic, which may lead to apparent suppression during electrospray MS analysis in the positive ion mode due to low ionization efficiency. These properties added to the general low abundance and substoichiometric ratio of phosphoryl peptides can result in certain peptides not being observed.

For three proteins, no tyrosine phosphorylation sites were identified. This implies that either the protein was co-immunoprecipitated by association to a tyrosine-phosphorylated protein or that the phosphorylated peptides were not detected due to one or more of the factors mentioned above.

### DISCUSSION

Overexpression of the FGFR-1 can lead to constitutive activation of the receptor and has been found in a variety of human cancers. Here we find that transient overexpression of the FGFR-1 in human 293 cells leads to a dramatic increase in the pool of Tyr(P)-containing proteins and activation of Erks (Fig. 1), implying constitutive activity of the overexpressed receptor. Anti-Tyr(P) immunoprecipitation together with MS precursor ion scanning was used in a tyrosine phosphoproteomic strategy to identify substrates, and their tyrosine phosphorylation sites, of FGFR-1 signaling. Precursor ion scanning for the im(Tyr(P)) (m/z 216.043) (18) proved to be a sensitive method for unbiased identification of in vivo tyrosine phosphorylation sites in tryptic digests of gel-separated proteins. 22 tyrosine phosphorylation sites were identified in six different proteins (see Table I), including several novel in vivo phosphorylation sites. We identified nine proteins in the FGFR-1 signaling pathway (Table I). These are substrates of the receptor itself or a downstream tyrosine kinase or alternatively complexed with a substrate protein. Two proteins have not previously been described as being involved in FGFR signaling: NSAP1 and TOM111. NSAP1 shares high sequence similarity with heterogeneous nuclear ribonucleoprotein R. A database search for conserved protein domains revealed three RNA recognition motifs, one of which covers Tyr-373 that was found to be phosphorylated in this study. RNA recognition motifs are a common feature in heterogeneous nuclear ribonucleoproteins, and the proteins are involved at many levels in the pre-mRNA and mRNA life cycle including transcriptional, splicing, nuclear export, and translational events (30). NSAP1 has been located to a complex bound to the major protein-coding region determinant of instability of c-Fos with influence on mRNA translation and degradation (31). A murine ortholog of NSAP1, Syncrip, was very recently found to be phosphorylated in response to insulin treatment of 3T3-L1 adipocytes (32). Phosphorylation of Syncrip was accommodated by the insulin receptor tyrosine kinase in vitro but was inhibited upon binding of RNA. Tyrosine phosphorylation at Tyr-373 in the third RNA recognition motif domain of

| Gel band in Fig. 1 | Protein | GI accession number | Tyr(P) localization |
|--------------------|---------|---------------------|--------------------|
| 1                  | PLCγ    | 4305869             | Tyr(P)-472, Tyr(P)-771, Tyr(P)-783, Tyr(P)-1253 |
| 2                  | FGFR-1  | 13186251            | Tyr(P)-152, Tyr(P)-278, Tyr(P)-305, Tyr(P)-461, Tyr(P)-581, Tyr(P)-583, Tyr(P)-603, Tyr(P)-651, Tyr(P)-652, Tyr(P)-728 |
| 3                  | p130CAS | 6740102             | Tyr(P)-128, Tyr(P)-249, Tyr(P)-306, Tyr(P)-327, Tyr(P)-410 |
| 4                  | HSP90   |                     |                    |
| 5                  | PAK5 p85 subunit | 12741979         |                    |
| 6                  | p90 ribosomal S6 kinase (RSK2) | 4759050     | Tyr(P)-707         |
| 7                  | Cortactin | 4885205              | Tyr(P)-446         |
| 8, 9               | NSAP1   | 14751580            | Tyr(P)-373         |
| 10                 | TOM111  | 4885639             |                    |

| Identified proteins and tyrosine phosphorylation sites |
|-------------------------------------------------------|
| Gel band in Fig. 1 | Protein | GI accession number | Tyr(P) localization |
|--------------------|---------|---------------------|--------------------|
| 1                  | PLCγ    | 4305869             | Tyr(P)-472, Tyr(P)-771, Tyr(P)-783, Tyr(P)-1253 |
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| 4                  | HSP90   |                     |                    |
| 5                  | PAK5 p85 subunit | 12741979         |                    |
| 6                  | p90 ribosomal S6 kinase (RSK2) | 4759050     | Tyr(P)-707         |
| 7                  | Cortactin | 4885205              | Tyr(P)-446         |
| 8, 9               | NSAP1   | 14751580            | Tyr(P)-373         |
| 10                 | TOM111  | 4885639             |                    |
FIG. 3. Identification of tyrosine phosphorylation sites by mass spectrometry. MS precursor ion scanning for the im(Tyr(P)) (m/z 216.043) to identify Tyr(P)-containing peptides. A, precursor ion scan of the complex peptide mixture generated by tryptic digestion of the protein excised from band 1 (Fig. 1). Peaks at m/z 655, 846, 904, and 993 (indicated by arrows) are indicative of a tyrosine-phosphorylated peptide. B, MS spectrum of the same region. Arrows indicate the position of the tyrosine-phosphorylated peptides observed in the precursor ion scan. C, MS/MS analysis of the doubly charged ions at m/z 655.3, 846.4, and 904.4 plus the triply charged ion at m/z 993.1. b- and y-ions are assigned as indicated. Sequence tags generated from the MS/MS spectra localized the tyrosine phosphorylation sites to Tyr-1253, Tyr-771, Tyr-783, and Tyr-472, respectively.
NSAP1/Syncrip can possibly influence its RNA binding properties and thus link FGFR-1 signaling to mRNA metabolism. TOM1l1 is an uncharacterized protein. A murine ortholog, SRCASM, was recently identified in a yeast two-hybrid screen for interaction partners of the Src family tyrosine kinase Fyn. The protein was found to interact with, and be a substrate of, the Fyn kinase upon co-transfection into COS cells. Sequence analysis revealed three putative SH2 binding consensus motifs for Grb2 SH2, phosphatidylinositol 3-kinase (PI3K) p85 subunit SH2, and Src/Fyn SH2 binding, respectively. From site-directed mutagenesis experiments, it appears that Tyr-457 in the Src/Fyn SH2 binding sequence is the major phosphorylation site. It also appeared, however, that PI3K and Grb2 bound to SRCASM in a manner that was dependent on phosphorylation of the tyrosine residues of their respective predicted SH2 binding sequences. However, tyrosine phosphorylation sites were not identified in TOM1l1. This is probably due to the lack of lysine and arginine residues in the region of the three proposed phosphorylation sites. Tryptic digestion yielded peptide fragments greater than 4.2 kDa, too large to be effectively analyzed by nanoelectrospray MS/MS. Mapping of the phosphorylation sites in the proteins identified is a very valuable means of validating the data. The possibility exists that a contaminating protein co-migrates with a relevant one in the SDS-PAGE and is subsequently identified by MS. To avoid false positives, we have minimized unspecific protein purification by using saturating conditions for immunoprecipitation and specific elution reagent. Furthermore, we have analyzed lysates from non-stimulated 293T cells stably transfected with the FGFR-1 (data not shown). Under conditions where no ligand-independent activation was observed we did not detect TOM1l1, and thus we propose TOM1l1 to be a new docking molecule in FGFR-1 signaling. The remaining seven identified proteins (Table I) have previously been described in signaling initiated by stimulation with fibroblast growth factors.

While we acquired no direct functional data for the role of the novel tyrosine phosphorylation sites, correlation with previous studies and sequence context predicts a functional role for several of the proteins as follows. We identified Tyr-707 in p90RSK2 as an in vivo phosphorylation site. p90RSK contains dual serine/threonine kinase domains. Exogenous substrate kinase activity is mediated by the N-terminal kinase domain, which is fully activated by the combined actions of Erks, PI3K-dependent kinase 1, and the C-terminal kinase domain of p90RSK (35–37). In a recent study it was found that a Y707A mutated variant of p90RSK exhibited constitutive activation independent of external stimuli or Erk activation (38). It is the authors’ hypothesis that a short C-terminal stretch (including the Tyr-707 residue) forms an autoinhibitory subunit that interferes with substrate binding to the catalytic site, similar to what has been described for the calcium/calmodulin-dependent protein kinase I (39). Upon truncation of this C-terminal stretch, or mutation of the Tyr-707 residue alone, autoinhibition is attenuated, and the kinase becomes constitutively active. Based on these findings we propose that phosphorylation of the Tyr-707 represents a novel alternative regulatory mechanism for p90RSK activation.

Five tyrosine phosphorylation sites were identified in p130CAS on Tyr-128, Tyr-249, Tyr-306, Tyr-327, and Tyr-410. These tyrosine residues are all located in the “substrate domain” of p130CAS that mediates binding to the SH2 domain of the adaptor molecule Crk. This domain has functionally been linked to actin dynamics in FGFR-1 signaling by Mass Spectrometry.
epidermal growth factor receptor (43), insulin-mediated (44), and integrin signaling (41). Cortactin, which is an actin-binding protein that also plays a role in actin cytoskeleton dynamics (45), was phosphorylated on Tyr-446 in our assay. Cortactin was first identified as a substrate of v-Src (46) that mediates in vitro phosphorylation of residues Tyr-421, Tyr-466, and Tyr-482 at the C terminus of the murine ortholog (47). Phosphorylation of these residues attenuates the F-actin cross-linking activity of cortactin but leads to an increase of events, such as cell migration, that are dependent on actin dynamics (47). We report here the identification of Tyr-446 as an in vivo phosphorylation site. Due to the localization of this tyrosine residue in the middle of the sequence containing the previously defined phosphorylation sites, we suggest a functional role in actin dynamics similar to that proposed by Huang and associates (47). Taken together the phosphorylation sites in p130CAS and cortactin represent interesting mechanisms by which overexpression of the FGFR-1 can possibly modulate actin cytoskeletal dynamics, cell migration, and possibly the invasion potential of tumors. Finally, we identified phosphorylation of tyrosine residues located in the extracellular region of the FGFR-1. A previous in vitro experiment indicated phosphorylation of two of these residues (Tyr-152 and Tyr-307) (48) in support of our data, but further experiments are required to determine how these phosphorylation sites modify the function of the receptor.

In this study we identified several proteins such as PLCγ and PI3K that have previously been described in the early stages of FGFR-1 signaling shortly after growth factor stimulation. It is possible, however, that some of the novel phosphorylation sites and proteins identified reflect events specific to sustained signaling. For example, FGF-1-stimulated tyrosine phosphorylation of cortactin has previously been described in the early stages of FGFR-1 signaling shortly after growth factor stimulation (49). Several of the proteins identified can functionally link FGFR-1 signaling to cellular events such as gene expression, actin cytoskeletal dynamics, and RNA processing. The tyrosine phosphorylation sites ascribed to these proteins in response to constitutive activation of the FGFR-1 may reveal the manner by which the receptor regulates these cellular processes.

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