Cell-surface ADP-ribosylation of fibroblast growth factor-2 by an arginine-specific ADP-ribosyltransferase

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Basic fibroblast growth factor (FGF-2) appeared to be ADP-ribosylated on the surface of adult bovine aortic arch endothelial and human hepatoma cells. Further characterization of this reaction with cells expressing an arginine-specific, glycosylphosphatidylinositol-anchored, mono-ADP-ribosyltransferase demonstrated that FGF-2 is ADP-ribosylated on arginine. Incubation of transformed cells with FGF-2 and [adenylate-³²P]nicotinamide-adenine dinucleotide (NAD) resulted in the rapid incorporation of [³²P]ADP-ribose into FGF-2 in a time- and concentration-dependent manner, with labelling averaging

3 mol of ADP-ribose/mol of FGF-2. Excess ADP-ribose had no effect on these reactions, whereas excess NAD inhibited the ADP-ribosylation of FGF-2, consistent with an enzymic rather than a non-enzymic ADP-ribosylation reaction. Heparin also inhibited the ADP-ribosylation reaction, whereas a neutralizing polyclonal anti-peptide antibody had no effect. Furthermore, the addition of putative receptor binding domain peptide analogues of FGF-2 reduced the maximal ADP-ribosylation of FGF-2. These results identify the cell-surface ADP-ribosylation of FGF-2 as a potentially ubiquitous event.

INTRODUCTION

Basic fibroblast growth factor (FGF-2) is a potent mitogen that induces new capillary growth in various pharmacological animal models, plays a role in the regeneration of tissues, and stimulates the healing of injured tissues [1–5]. The proliferation state in normal, uninjured vascular endothelia is generally low, even though significant quantities of FGF-2 are usually present [6]. Further, FGF-2, known to be necessary and sufficient for initiation of angiogenesis, must be under rigorous control, since unrestrained angiogenesis can lead to pathological conditions such as atherosclerosis, rheumatoid arthritis and diabetic retinopathy. Although the tight association of FGF-2 with basement membranes and its strong affinity for heparin regulates its bioavailability and controls its interaction with its high-affinity receptors, FGF-2 activity also may be regulated by post-translational modification. FGF-2 is both phosphorylated and ADP-ribosylated *in vitro* [7–10], and we sought to determine if FGF-2 could be ADP-ribosylated on the surface of cultured cells.

The eukaryotic ADP-ribosylation system was characterized originally by its ability to modify proteins, not only by transfer of a single ADP-ribose (ADPR) residue from β -nicotinamide-adenine dinucleotide (NAD), but also by the removal of ADPR groups, resulting in alteration of the target proteins' functional properties [11–14]. This ADP-ribosylation cycle involves amino acid-specific ADPR transferases and ADPR-arginine hydrolases [15–20]. Cell surface ADP-ribosyltransferases have been identified in many tissues, including liver, thyroid and pancreatic islets of Langerhans [21–23]. In peripheral tissues, specific hormones and second-messenger systems regulate endogenous ADP-

ribosylation [24–27]. A recently cloned and purified arginine-specific, mono-ADP-ribosyltransferase is anchored to the cell surface by a glycosylphosphatidylinositol tail (GPI-anchored) [18,19]. Interestingly, the mRNA encoding this particular cell surface-associated ADP-ribosyltransferase is detected primarily in skeletal and heart muscle, both of which contain significant amounts of FGF-2 and are targets of FGF-2 action [28–30].

We describe here the ADP-ribosylation of FGF-2 by cultured adult bovine aortic arch endothelial (ABAE) cells, human hepatoma (SK-Hep1) cells, and by rat adenocarcinoma (NMU) cells transformed with a cDNA encoding a GPI-anchored ADP-ribosyltransferase (pM-T). Our studies demonstrate that the reaction is inhibited by heparin and specific peptides, but not by ADPR or a polyclonal antibody raised against a peptide corresponding to the N-terminus of FGF-2. We propose that the ADP-ribosylation of FGF-2 may provide an additional level of control of FGF-2 activity.

MATERIALS AND METHODS

Materials

Recombinant human FGF-2 (FGF-2, 18 kDa form) was produced in our laboratory by expression in *Escherichia coli*. NMU cells transformed with a mammalian expression vector containing the cDNA for the rabbit skeletal muscle ADP-ribosyltransferase (pM-T) or mock-transfected with the vector only (pM) were generously given by Dr. Joel Moss (Bethesda, MD, U.S.A.). [adenylate-³²P]NAD (800 Ci/mmol) was purchased from DuPont NEN (Boston, MA, U.S.A.). Peptides were synthesized by solid-phase methodology [31]. The peptide fragments of

Abbreviations used: ABAE cells, adult bovine aortic arch endothelial cells; ADPR, ADP-ribose; FGF-2, basic fibroblast growth factor; FGFR, basic fibroblast growth factor receptor; GPI, glycosylphosphatidylinositol; NAD, nicotinamide-adenine dinucleotide; NMU cells, rat adenocarcinoma cells; SK-Hep1 cells, human hepatoma cells.

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FGF-2 correspond to the bovine sequence with the amino acid numbering of FGF-2 described for the 146-amino-acid form [32]. Neutralizing antibody 773 is an antiserum against FGF-2(1–24) conjugated to BSA [8,33,34]. Dexamethasone sodium phosphate was purchased from Spectrum Chemicals (Gardena, CA, U.S.A.), reagents for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.), X-OMAT films from Eastman Kodak (Rochester, NY, U.S.A.), and all other reagents from Sigma (St. Louis, MO, U.S.A.).

Cell culture and transferase expression

ABAE cells and SK-Hep1 cells were grown to confluence in 15-cm plates in Dulbecco's Modified Eagle's Medium (Gibco, Bethesda, MD, U.S.A.) containing 1 g/litre (ABAE cells) or 4.5 g/litre (SK-Hep1 cells) glucose and supplemented with calf (ABAE cells) or 10% fetal (SK-Hep1 cells) serum. FGF-2 (at 2 ng per plate of ABAE cells) was added every other day to the culture media to maintain the cells in exponential growth. pM-T- or pM-transfected mammary NMU cells (pM-T or pM) were grown in Eagle's Minimum Essential Medium in Earle's Balanced Salt Solution with L-glutamine (BioWhittaker, Walkersville, MD, U.S.A.), 10% heat-inactivated fetal-calf serum and Geneticin (G418, 0.5 mg/ml). Transferase expression was induced by incubating cells with dexamethasone sodium phosphate (Spectrum Chemical Manufacturing, Gardena, CA, U.S.A.) (1 μ M) for 24 h.

Cell-surface ADP-ribosylation

To evaluate ADP-ribosylation of FGF-2, intact ABAE, SK-Hep1, pM-T or pM cells were prepared at a density of 50000 cells/well (ABAE, pM and pM-T) or 30000 cells/well (SK-Hep1) in 24-well plates and incubated for 2–4 days without change of medium. At confluence, cells were washed three times with Dulbecco's phosphate-buffered saline (Gibco). Reaction mixes were prepared in Eppendorf tubes containing 100 μ l of a reaction buffer (50 mM Tris/HCl, pH 8.0/2 mM MgCl₂/1 mM CaCl₂/3 mM dithiothreitol), FGF-2 and/or other components, as indicated in each Figure legend. Reaction mixtures were added to each well and the reactions were initiated with the addition of [adenylate-³²P]NAD (10 μ Ci; 800 Ci/mmol). Supernatants were removed from the cells at the appropriate time and immediately added to sample buffer [35] to stop the reactions. The samples were resolved by SDS/PAGE [15% (w/v) acrylamide], transferred to nitrocellulose, and visualized by autoradiography (X-OMAT film; 1–4 day exposure at -80°C). Autoradiography exposure times for samples from pM-T and pM cells were < 1 h at -80°C . In some experiments, the band corresponding to labelled FGF-2 was excised and radioactivity was counted in a β -counter, without scintillation fluid.

RESULTS

ADP-ribosylation of FGF-2 in ABAE and SK-Hep1 cells

We chose cell lines used extensively in this laboratory in other studies of FGF-2 activity, location and mode of action, to study the potential application of ADP-ribosylation as a post-translational modification of FGF-2. ABAE cells require FGF-2 for continued proliferation in culture and probably represent a typical cell type that would respond to delicate nuances in FGF-2 activity [36–38], whereas SK-Hep1 cells naturally produce high levels of FGF-2 [7–9]. Both cell types also represent tissues with ADP-ribosyltransferase activity.

Incubation of cells in the presence of FGF-2 and [adenylate-³²P]NAD indicated incorporation of [adenylate-³²P]ADP-ribose

Figure 1 Incorporation of radiolabel from [³²P]NAD into FGF-2

Dose–response for ADP-ribosylation of FGF-2: (A) ABAE cells; (B) SK-Hep1 cells. ABAE (50000 cells/well) or SK-Hep1 (30000 cells/well) cells were incubated for 60 min at room temperature in the presence of a reaction mix consisting of 100 μ l of 50 mM Tris/HCl, pH 8.0, 2 mM MgCl₂, 1 mM CaCl₂, 3 mM dithiothreitol and the indicated amounts of FGF-2 and [adenylate-³²P]NAD (10 μ Ci/well). After incubation, the cell supernatants were collected into Eppendorf tubes containing Laemmli sample buffer [43] to stop the reactions, divided in half and analysed for the presence of radiolabel by SDS/PAGE (15% acrylamide), by transfer to nitrocellulose, and by autoradiography, as described in the Materials and methods section. The arrow highlights the band corresponding to FGF-2. Time course for ADP-ribosylation of FGF-2 with [³²P]NAD [(A) ABAE cells; (B) SK-Hep1 cells]: ABAE (50000 cells/well) or SK-Hep1 (30000 cells/well) cells were incubated with 1 μ g of FGF-2 for varying times at room temperature in the presence of a reaction mix consisting of 100 μ l of reaction buffer and [adenylate-³²P]NAD (10 μ Ci/well); samples were processed as described in the Materials and methods section.

FGF-2 by both cell types. This reaction was dose- and time-dependent, with an apparent K_m of 13.3 nM and with at least 3 mol of ADP-ribose incorporated per mol of FGF-2 (Figure 1).

We added varying concentrations of unlabelled NAD to the reactions containing FGF-2 and [adenylate-³²P]NAD. The unlabelled NAD reduced the extent of [³²P]ADP-ribose incorporation into FGF-2, yet unlabelled ADP-ribose had no effect on the reaction (Figure 2). Therefore we conclude that the reaction involves the enzymic addition of ADP-ribose, as opposed to protein glycation by free ADP-ribose generated from the degradation of NAD by NAD glycohydrolases [39–41].

Effect of ADP-ribosylation on the interaction of FGF-2 with target cells

To determine if ADP-ribosylation of FGF-2 affects its ability to interact with the cell surface, we added varying concentrations of heparin (for which FGF-2 has a high affinity [6,42]) to the reaction mixture. Interestingly, heparin inhibited the extent of incorporation of [adenylate-³²P]ADP-ribose into FGF-2, implying that heparin binding of FGF-2 versus ADP-ribosylation of FGF-2 are mutually exclusive reactions (Figures 3A and 3B). The neutralizing antibody 773 inhibits the interaction between FGF-2 and its high-affinity receptor on ABAE and SK-Hep1 cells [7,8,43]. However, this antibody has no effect on FGF-2 ADP-ribosylation by either ABAE or SK-Hep1 cells (Figure 3), which is evidence that ADP-ribosylation of FGF-2 can occur without its binding to its high-affinity cell-surface receptor.

Figure 2 Cell-surface-mediated ADP-ribosylation of FGF-2 is inhibited by NAD, but not by ADP-ribose

ABAE (50 000 cells/well) or SK-Hep1 (30 000 cells/well) cells were incubated with a reaction mix consisting of 100 μ l of reaction buffer, 1 μ g of FGF-2, [*adenylate*- 32 P]NAD (10 μ Ci/well) and varying amounts of NAD (**A**) and (**B**) or ADP-ribose (**A**) and (**C**) for 60 min at room temperature [NAD was diluted from a 13 mM stock (in PBS), and 10 pmol, 1 nmol or 100 nmol was added to the reaction mix; ADP-ribose was diluted from a 1.788 mM stock (in PBS) and 10 pmol, 1 nmol or 100 nmol was added to the reaction mix]. Supernatants were removed from the cells at 60 min and immediately added to sample buffer to stop the reactions. Samples were processed as described in the Materials and methods section. (FGF-2 = positive control.) (**A**) ABAE cells; (**B**) and (**C**) SK-Hep1 cells. The arrow indicates the band corresponding to FGF-2.

Putative site of ADP-ribosylation on FGF-2

We used a series of peptides corresponding to fragments of FGF-2 to identify the region of FGF-2 that was subject to ADP-ribosylation, all of which had the same effect on the reaction catalysed by either ABAE or SK-Hep1 cells. A peptide whose sequence corresponds to the putative receptor-binding domain of FGF-2 [FGF-2-(106–120)NH₂] inhibited ADP-ribosylation of FGF-2, whereas another peptide, whose sequence corresponds to another region of FGF-2 [FGF-2-(139–146)NH₂], had no effect on the extent of ADP-ribosylation of FGF-2 (Figure 4A). These data indicate that the target amino acid of FGF-2 modified by ADP-ribosylation may be in its receptor-binding domain, which contains three arginine residues (R-S-R-K-Y-T-S-W-Y-V-A-L-K-R-T).

ADP-ribosylation of FGF-2 by a GPI-linked arginine-specific mono-ADP-ribosyltransferase

The isolation, characterization and subsequent cloning of an arginine-specific ADP-ribosyltransferase anchored in the cell membrane by a GPI tail [19,20], led us to study the ADP-ribosylation of FGF-2 NMU cells transformed with the rabbit skeletal muscle ADP-ribosyltransferase (pM-T cells). The extra-cellular location of the ADP-ribosyltransferase seemed logical

Figure 3 Cell-surface-mediated ADP-ribosylation of FGF-2 is inhibited by heparin, but not by a polyclonal peptide antibody

ADP-ribosylation of FGF-2 was inhibited by increasing concentrations of heparin. Briefly, ABAE (50 000 cells/well) or SK-Hep1 (30 000 cells/well) cells were incubated with a reaction mix consisting of 100 μ l of reaction buffer, 1 μ g of FGF-2, [*adenylate*- 32 P]NAD (10 μ Ci/well) and varying amounts of heparin, and analysed as described in the Materials and methods section. The polyclonal peptide antibody 773 was incubated with 1 μ g of FGF-2 for 15 min at room temperature and added to ABAE (50 000 cells/well) or SK-Hep1 (30 000 cells/well) cells with [*adenylate*- 32 P]NAD (10 μ Ci/well). Controls: '0', no FGF-2; 'FGF-2', FGF-2 only. (**A**) ABAE cells; (**B**) and (**C**) SK-Hep1 cells. The arrow indicates the band corresponding to FGF-2.

Figure 4 Effect of FGF-2 peptides on cell-surface-mediated ADP-ribosylation of FGF-2

ABAE (50 000 cells/well) or SK-Hep1 (30 000 cells/well) cells were incubated with a reaction mix consisting of 100 μ l of reaction buffer, 1 μ g of FGF-2 and either peptide FGF-2-(106–120)NH₂ (106–120) or peptide FGF-2-(139–146)NH₂ (139–146). Peptides (10 μ g) were prepared by solubilization in 5 mM HCl before addition to assay mixes. The addition of [*adenylate*- 32 P]NAD (10 μ Ci/well) initiated the reactions, and samples were analysed as described in the Materials and methods section. (**A**) ABAE cells: lane 1, peptide FGF-2-(106–120)NH₂; lane 2, peptide FGF-2-(139–146)NH₂; lane 3 (0), no FGF-2; lane 4 (FGF-2), no peptide. (**B**) SK-Hep1 cells: lane 1 (FGF-2), no peptide; lane 2, peptide FGF-2-(106–120)NH₂; lane 3, peptide FGF-2-(139–146)NH₂; lane 4 (0), no FGF-2. The arrow indicates the band corresponding to FGF-2. Lanes are numbered from the left.

Figure 5 ADP-ribosylation of FGF-2 on the surface of cells expressing a GPI-linked, arginine-specific, mono-ADP-ribosyltransferase

Reactions were carried out as described for Figures 1–4 and in the Materials and methods section. **(A)** Dose–response: the concentrations of FGF-2 for each reaction were as follows: lane 1, 0 ng; lane 2, 50 ng; lane 3, 100 ng; lane 4, 200 ng; lane 5, 500 ng; lane 6, 1 μg; lane 7, 2 μg; lane 8, 4 μg. The bands corresponding to labelled FGF-2 were excised and counted in a β-counter. Time course of FGF-2 ADP-ribosylation: Incubation times for each reaction were as indicated. **(B)** Inhibition of ADP-ribosylation of FGF-2 in the presence of NAD, but not ADP-ribose. **(C)** Effect of heparin on the extent of labelling of FGF-2. **(D)** Peptides corresponding to internal sequences of FGF-2 were included in (lanes 2–6) or excluded from (lanes 1 and 7) ADP-

for a regulatory enzyme involved in the modulation of FGF-2 activity.

FGF-2 was clearly ADP-ribosylated by the arginine-specific ADP-ribosyltransferase. This reaction was dose- and time-dependent (Figure 5A). These results exactly mimic those obtained with both the ABAE and SK-Hep1 cells. However, the reaction is more efficient on the surface of pM-T-transfected cells than on the surfaces of either ABAE or SK-Hep1 cells. With comparable cell number, reaction time and concentrations of FGF-2 and [adenylate-³²P]NAD, the time required to detect a signal by autoradiography for the pM-T-transfected NMU cells was less than 1 h. By contrast, exposure times to detect a signal for the ABAE and SK-Hep1 cell experiments were 1–4 days. These data indicate the specificity of the GPI-anchored ADP-ribosyltransferase and the efficiency of the reaction on the transfected cells, and possibly reflect a higher concentration of cell-surface transferase molecules. Reactions with mock-transfected pM cells revealed non-detectable signals under comparable conditions (results not shown).

Specificity of the GPI-linked ADP-ribosyltransferase for FGF-2

The addition of NAD and ADP-ribose to pM-T cells expressing the GPI-anchored ADP-ribosyltransferase in the presence of FGF-2 and [³²P]NAD effectively inhibited the labelling of FGF-2 or had no effect respectively, mirroring the results obtained with ABAE and SK-Hep1 cells (Figure 5B). Correspondingly, the addition of heparin to the reactions decreased the extent of labelling of FGF-2 in a dose-dependent manner (Figure 5C).

Finally, only one [FGF-2-(93–120)] of various peptides corresponding to specific sequences of the FGF-2 molecule affects the ADP-ribosylation of FGF-2 on the surface of pM-T cells. FGF-2-(93–120) also inhibited the ADP-ribosylation of FGF-2 in the presence of ABAE and SK-Hep1 cells (results not shown). We attribute this inhibition to the fact that the peptide itself is a substrate for the GPI-anchored ADP-ribosyltransferase (since it contains four arginine residues), thus effectively preventing it from inhibiting the ADP-ribosylation of FGF-2. This inhibition may also be the result of the peptide blocking access of FGF-2 to the cell surface and therefore to the ADP-ribosyltransferase activity. Peptide FGF-2-(106–120) does not inhibit the ADP-ribosylation of FGF-2 on the surface of pM-T cells, in contrast with results on ABAE and SK-Hep1 cells (Figure 5D). There is a high probability that the presence of the highly effective concentration of GPI-anchored ADP-ribosyltransferase activity on the pM-T cells can overcome the inhibitory effect seen with the ABAE and SK-Hep1 cells.

DISCUSSION

In this study, we show that a reaction in which FGF-2 is ADP-ribosylated on the surface of ABAE and SK-Hep1 cells also occurs in the presence of rat NMU cells expressing a cloned, arginine-specific, ADP-ribosyltransferase anchored to the cell membrane by a GPI tail. This reaction is dose- and time-dependent and appears to be enzymic, since ADP-ribose (in 100-fold excess over the [³²P]NAD in the reaction) has no effect on

ribosylation reactions catalysed by confluent dexamethasone-treated pM-T-transformed cells (50 000 cells/well). Lane 1 (FGF-2), no peptide; lane 2, peptide FGF-2-(93–120)NH₂; lane 3, peptide FGF-2-(106–120)NH₂; lane 4, peptide [R108S]FGF-2-(106–120)NH₂, which is peptide FGF-2-(106–120)NH₂ with a point mutation at amino acid 108; lane 5, peptide [K109R]FGF-2-(106–120)NH₂, which is peptide FGF-2-(106–120)NH₂ with a point mutation at amino acid 109; lane 6, peptide FGF-2-(139–146)NH₂; lane 7 (0), contains no FGF-2.

the incorporation of [32 P]ADP-ribose from [32 P]NAD into FGF-2. Peptides whose amino acid sequences correspond to the putative receptor-binding domain of FGF-2 not only inhibited the reaction, but also were themselves substrates for ADP-ribosylation, and inhibition may have resulted, in part, from their ability to serve as alternative substrates for the transferase.

FGF-2 has an extracellular receptor and, because of its relatively high affinity for heparin, is localized and possibly sequestered by heparan sulphates on the cell surface and in the extracellular matrix [44–46]. There are large quantities of FGF-2 in tissues that do not appear to be active and that are not 'released' from the cell surface; however, when FGF-2 is bound to glycosaminoglycans, it appears to be protected from degradation [47–50]. The high-affinity binding of FGF-2 to heparin may also block its availability to the surface transferase, thereby preventing ADP-ribosylation. FGF-2 is transferred from its low-affinity receptors to its high-affinity receptor (FGFR) located in the plasma membrane of endothelial cells and modulates cell proliferation in two apparently distinct events: internalization with transport of the FGF/FGFR complex to the nucleus, and activation of the autophosphorylating tyrosine kinase domain of the receptor. Heparin inhibits the ADP-ribosylation of FGF-2, and a polyclonal antibody against FGF-2 has no effect on the reaction. We take these results to mean that ADP-ribosylation may inhibit the low-affinity binding of FGF-2. This subtle change in binding affinity could have profound effects on FGF-2 availability to the cell.

FGF-2 is also phosphorylated, presumably by a cell-surface-associated kinase, and this phosphorylation affects its activity as well as its interaction with its high- and low-affinity receptors on the cell surface [7–9]. These results suggest that a delicate balance exists between the binding of FGF-2 to its cell-surface receptors, its activation by phosphorylation, and the potential modulation of FGF-2 activity by ADP-ribosylation. These phenomena together would exhibit the fine-tuning mechanisms expected in the regulation of such a potent mitogen.

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