Participation of Na,K-ATPase in FGF-2 Secretion: Rescue of Ouabain-Inhibitable FGF-2 Secretion by Ouabain-Resistant Na,K-ATPase α Subunits†

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ABSTRACT: We have examined the relationship between Na,K-ATPase and FGF-2 secretion in transfected primate cells. FGF-2 lacks a classic hydrophobic export signal, and the mechanisms mediating its secretion are unknown. To monitor secretion, a FLAG epitope tag was inserted into the carboxyl terminus of the 18 kDa form of human FGF-2, and the construct was transfected into either human HEK 293 or monkey CV-1 cells. Exported FGF-2 was detected in the culture medium using the FLAG-specific monoclonal antibody M2. FGF-2 secretion from HEK 293 or CV-1 cells was linear over time and sensitive to inhibition by the cardiac glycoside ouabain, a specific inhibitor of the Na,K-ATPase. In contrast, the secretion of FGF-8 (an FGF family member that contains a hydrophobic secretory signal) was not inhibited by treatment of HEK 293 or CV-1 cells with ouabain. FGF-2 secretion was also assayed in CV-1 cells expressing the naturally ouabain-resistant rodent Na,K-ATPase α1 subunit. In cells expressing the rodent α1 subunit, FGF-2 secretion was unaffected by high levels of ouabain, indicating that the rodent α1 subunit was capable of rescuing ouabain-inhibitable FGF-2 export. Expression of ouabain-resistant mutants of the rodent α2 and α3 subunits, or the naturally ouabain-resistant rodent α4 subunit, also supported FGF-2 secretion in ouabain-treated cells. Taken together, our studies are consistent with the idea that the Na,K-ATPase plays a prominent role in regulating FGF-2 secretion, although none of the α subunit isoforms exhibited specificity with regard to FGF-2 export.

The Na,K-ATPase is an integral membrane protein responsible for maintaining the electrochemical gradient across the plasma membrane of virtually all mammalian cells. The Na,K-ATPase performs this function by catalyzing the exchange of intracellular sodium ions for extracellular potassium ions, a process that is coupled to ATP hydrolysis (*1*). The holoenzyme is composed of equimolar amounts of α and *β* subunits (*2*). The α subunit contains multiple membrane-spanning segments that anchor the polypeptide in the plasma membrane. The α subunit also contains the catalytic site for ATP hydrolysis and is the cellular receptor for the cardiac glycoside class of cardiotonic drugs such as ouabain and digitalis (*1*, *3*). The *β* subunit is a type II membrane protein containing a single transmembrane segment and is extensively glycosylated. The *β* subunit is an essential component of the Na,K-ATPase, although its exact role in Na,K-ATPase function has not yet been elucidated.

Molecular cloning has revealed the existence of four α and three *β* subunit isoforms in both humans and rodents (*4*−*6*). Within each species, the α subunits are highly conserved (75−85% identical), whereas the *β* subunits show much greater sequence diversity (35−40% identical). In mice and humans, each of the α and *β* subunits is encoded by a separate gene, and each gene exhibits a distinct tissue-specific expression profile (*5*). Chromosomal dispersion and differing expression patterns suggest specialized functional roles for each of the α and *β* isoforms. Expression of α and *β* subunit cDNAs in heterologous systems indicates that α−*β* subunit interaction is promiscuous, and that all possible α−*β* combinations could potentially associate to form active holoenzyme (*7*−*9*). The functional significance of this high degree of isoenzyme heterogeneity is not currently understood.

Recently, Florkiewicz et al. (*10*) made the remarkable observation that cardiac glycosides inhibited the secretion of fibroblast growth factor-2 (FGF-2)[1] from transfected COS-1 cells. FGF-2 is a member of the FGF family of mitogenic peptides that is currently known to be comprised of at least 18 distinct members with a wide variety of biological activities (*11*). FGF-2 has been shown to play an important role in cardiogenesis (*12*), liver development (*13*), and angiogenesis (*14*). The majority of FGFs contain a hydrophobic leader sequence that targets the polypeptides for secretion via the endoplasmic reticulum (ER)/Golgi pathway. However, FGF-1, FGF-2, and FGF-9 lack the hydrophobic export signal (*11*), and determining the exact mechanism regulating their secretion has proven to be elusive. The inhibition of FGF-2 secretion by cardiac glycosides suggests that FGF-2 is released from cells by a novel secretory mechanism involving Na,K-ATPase (*10*).

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[1] Abbreviations: FGF, fibroblast growth factor; ER, endoplasmic reticulum; EST, expressed sequence tag; ORF, open reading frame; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody.

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To further characterize the role of Na,K-ATPase in mediating FGF-2 secretion, we have developed an assay system that allows us to monitor the release of epitope-tagged FGF-2 from transfected primate cells using an epitope tag-specific monoclonal antibody (mAb). Using this assay, we show that FGF-2 secretion is inhibited by ouabain, but can be restored by expression of ouabain-resistant Na,K-ATPase α subunits. Each of the four Na,K-ATPase α subunit isoforms appears to be capable of rescuing ouabain-inhibitable FGF-2 export, indicating that none of the α subunit isoforms has evolved specifically to regulate FGF-2 secretion. The Na,K-ATPase α subunit and FGF-2 can be co-immunoprecipitated from transfected cells, indicating the potential for interaction between the two polypeptides. The ability of the Na,K-ATPase α subunit to rescue FGF-2 export in ouabain-poisoned cells provides formal genetic proof that the Na,K-ATPase participates in the pathway governing FGF-2 secretion. This involvement suggests a physiological function for Na,K-ATPase in addition to its role in ion transport.

EXPERIMENTAL PROCEDURES

DNA Constructs and Transfections. An EST (IMAGE Consortium clone 172276; GenBank accession number H19554) containing the complete ORF (open reading frame) for human FGF-2 was obtained from Genome Systems (St. Louis, MO). A cDNA containing the complete ORF for murine FGF-8 (ATCC 63351; GenBank accession number Z46883) was obtained from American Type Culture Collection (Manassas, VA). PCR mutagenesis (*15*) was used to generate the 18 kDa secreted form of FGF-2 (*16*) by truncating the 5′ end of FGF-2 cDNA and introducing a perfect Kozak sequence (*17*) six nucleotides before the initiating methionine. This procedure removed the upstream translation initiation sites that produce the unsecreted, high-molecular mass forms of FGF-2 (*18*, *19*). FLAG epitope tags were inserted at the carboxyl termini of FGF-2 and FGF-8 cDNAs by PCR mutagenesis as described by Nelson and Long (*15*). Each tagged polypeptide was verified by DNA sequencing and then subcloned into the eukaryotic expression vector pCB6 (*20*).

Monkey CV-1 or human embryonic kidney (HEK) 293 cells were used as recipients for transfection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were performed using the Effectene transfection reagent (Qiagen) according to instructions supplied by the manufacturer. Cells transiently transfected with FLAG-tagged FGF-2 or FGF-8 constructs were maintained in medium supplemented with 20 μg/mL heparin. CV-1 and HEK 293 cells stably expressing either the naturally ouabain-resistant rat α1 or rat α4 subunits were produced as previously described (*21*, *22*). Mutated, ouabain-resistant forms of the rat α2 and α3 subunits were used in transfection to generate stable ouabain-resistant CV-1 and HEK 293 cell lines by the methods described elsewhere (*23*, *24*).

Cell viability was assessed by the trypan blue exclusion method as described previously (*25*). The viability of cells cultured in drug-free medium was compared to that of cells grown for 4 h in the presence of 100 μM ouabain. The increase in the number of nonviable cells in ouabain-treated cultures never exceeded by more than 2% (5.25 vs 3.5%) the number of nonviable cells in cultures grown in drug-free medium.

FGF Secretion Assay. CV-1 or HEK 293 cell lines were separately transfected with FLAG-tagged FGF-2 or FGF-8 constructs as described. Cells were split in a 1:6 ratio 24 h after transfection, and then grown for an additional 24 h prior to initiating secretion assays. To assess secretion, cells were washed for 1 min with 100 mM Na_2CO_3, and the medium was replaced with DMEM, DMEM containing 100 μM ouabain, or DMEM containing 25 μg/mL Brefeldin A (Sigma). Cells were grown in the presence or absence of drug for 4 h. Medium was collected and cleared by centrifugation at 3000 rpm for 10 min at 4 °C. Aliquots of medium (25 μL) were fractionated on SDS-containing 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, and the filters were blocked for 1 h in phosphate-buffered saline (PBS) containing 10% dry milk and 0.1% Tween 20. Filters were incubated with a 1:500 dilution of the FLAG-specific M2 mAb (Sigma) and proteins visualized using horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Immunoreactivity was detected by enhanced chemiluminescence (ECL) using an ECL Plus kit (Amersham). Western blots were quantitated using a laser densitometer (Molecular Dynamics) and analyzed using the Quantity One software package (PDI, Inc.). Data were subjected to a one-tailed Student's *t* test.

Immunoprecipitation. For immunoprecipitation, cell lysates were prepared from either transfected or mock-transfected cells using protocols described in the ImmunoCatcher immunoprecipitation kit (CytoSignal, Irvine, CA). Cell lysates were incubated overnight at 4 °C with either a 1:250 dilution of anti-Na,K-ATPase α1 subunit mAb (Upstate Biotechnology) or a 1:200 dilution of an anti-FLAG polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein A/G resin (Cytosignal) was used to capture antigen, and bound proteins were eluted with SDS−PAGE gel loading buffer. Proteins were fractionated by electrophoretic separation on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and detected with one of the following probes: (a) 1:1000 dilution of the anti-FLAG polyclonal antibody, (b) 1:500 dilution of the anti-FLAG M2 mAb, and (c) 1:1000 dilution of an anti-FGF-2 mAb (Upstate Biotechnology).

RESULTS

FGF-2 Secretion from Transfected Primate Cells. In an initial series of experiments, we examined the utility of epitope tagging for detection of exported FGF-2 from transfected cells. To monitor secretion, a human FGF-2 cDNA carrying a carboxyl-terminal FLAG epitope tag was transiently expressed in either CV-1 or HEK 293 cells. Aliquots of culture medium were then assayed for the presence of FGF-2 at various times after transfection. As shown in Figure 1A, FLAG-tagged FGF-2 secreted from CV-1 cells was readily detected using the FLAG-specific mAb M2. FLAG-tagged FGF-2 could also be detected in the culture medium taken from transiently transfected HEK 293 cells (data not shown). These results indicate that CV-1 and HEK 293 cells are capable of supporting FGF-2 export

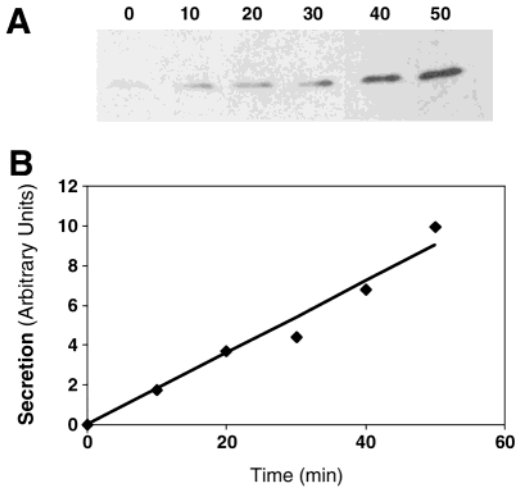

FIGURE 1: Secretion of FLAG-tagged FGF-2 from transfected CV-1 cells. Aliquots of medium were collected every 10 min from FGF-2-transfected cells. Proteins were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the anti-FLAG M2 mAb. (A) Western blot. (B) Quantitation of bands by laser densitometry. The best fit of the data was determined by linear regression analysis ($R^2 = 0.9447$).

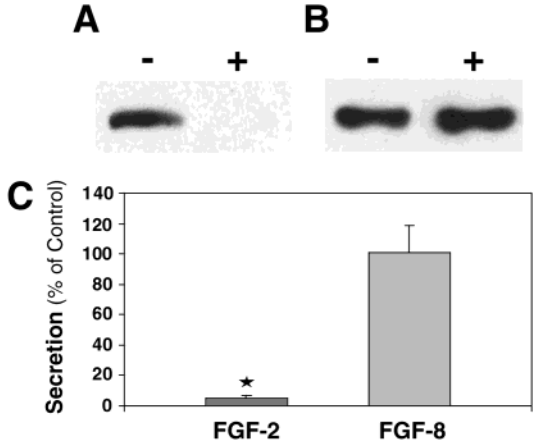

FIGURE 2: Ouabain inhibits FGF-2, but not FGF-8, secretion from CV-1 cells. Aliquots of medium were collected from CV-1 cells transiently transfected with FLAG-tagged FGF-2 (A) or FGF-8 (B) constructs. Cells were cultured in the presence (+) or absence (−) of 100 μM ouabain. Proteins were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed using the anti-FLAG M2 mAb. (A and B) Western blot. (C) Quantitation of bands by laser densitometry. Bars represent the data obtained from two transfection experiments repeated in duplicate. Error bars represent the standard error of the mean (SEM). The asterisk indicates statistically significant reduction of the level of FGF-2 export in the presence of 100 μM ouabain ($p < 0.005$).

and that epitope tagging provides a convenient method for monitoring FGF-2 secretion from transfected cells. Using this approach, we found that FGF-2 secretion from CV-1 cells was linear over a 50 min time period (Figure 1B), supporting the view that the secretion of FGF-2 occurs in a regulated fashion.

We next examined the effect of ouabain on FGF-2 export from primate cells. Ouabain is a specific inhibitor of the Na,K-ATPase, and CV-1 and HEK 293 cells are known to express a ouabain-sensitive α1 subunit isoform (*21*, *26*). As shown in panels A and C of Figure 2, a dose of 100 μM ouabain was effective in reducing the level of FGF-2 export

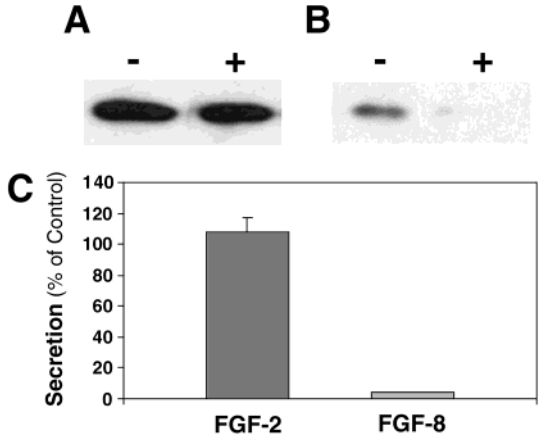

FIGURE 3: Brefeldin A inhibits FGF-8, but not FGF-2, secretion from CV-1 cells. Aliquots of medium were collected from CV-1 cells transiently transfected with FLAG-tagged FGF-2 (A) or FGF-8 (B) constructs. Cells were cultured in the presence (+) or absence (−) of 25 μg/mL Brefeldin A. Proteins were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the anti-FLAG M2 mAb. (A and B) Western blot. (C) Quantitation of bands by laser densitometry. The bar for FGF-2 represents the data obtained from two separate transfections each performed in duplicate. The bar for FGF-8 represents the data obtained from one transfection experiment. The error bar represents the SEM.

from CV-1 cells by >90% compared to control cells. Similar results were obtained using HEK 293 cells (data not shown). In contrast, 100 μM ouabain had virtually no effect on the export of FLAG-tagged FGF-8 from either CV-1 (Figure 2B,C) or HEK 293 cells (data not shown). FGF-8 contains a hydrophobic signal sequence and is believed to be secreted via the ER/Golgi pathway (*27*). The ability of ouabain-treated CV-1 cells to support FGF-8 secretion suggests that the inhibitory effect of ouabain on FGF-2 secretion does not result from a nonspecific effect of the drug on protein secretion. Brefeldin A (an inhibitor of the ER/Golgi pathway) (*28*) blocked the export of FGF-8 (Figure 3B,C), but not FGF-2 (Figure 3A,C), from transfected CV-1 cells. These studies indicate that FGF-2 and FGF-8 utilize different secretory pathways for release into the extracellular environment.

To analyze the effect of ouabain on FGF-2 secretion, we compared FGF-2 expression in cells grown in the presence or absence of ouabain. As shown in Figure 4A, treatment of cells with ouabain did not cause any noticeable degradation of FGF-2. In fact, exposure of cells to ouabain resulted in an apparent increase in intracellular FGF-2 levels. Light microscopic examination of FGF-2 expression is consistent with the view that ouabain inhibition of FGF-2 secretion caused an increase in the cytsolic levels of FGF-2. In control cells, FGF-2 immunoreactivity was detected primarily in association with cell margins (Figure 4B). In cells treated with ouabain, however, there was a noticeable increase in the level of cytosolic FGF-2 immunoreactivity (Figure 4C). These results strongly suggest that ouabain does not block secretion via intracellular degradation of the FGF-2 polypeptide. Taken together, our data are consistent with the idea that ouabain specifically blocks FGF-2 secretion, and that the Na,K-ATPase plays an important role in mediating cellular FGF-2 export.

To further analyze the role of Na,K-ATPase in FGF-2 export, we assessed the ability of a ouabain-resistant α1

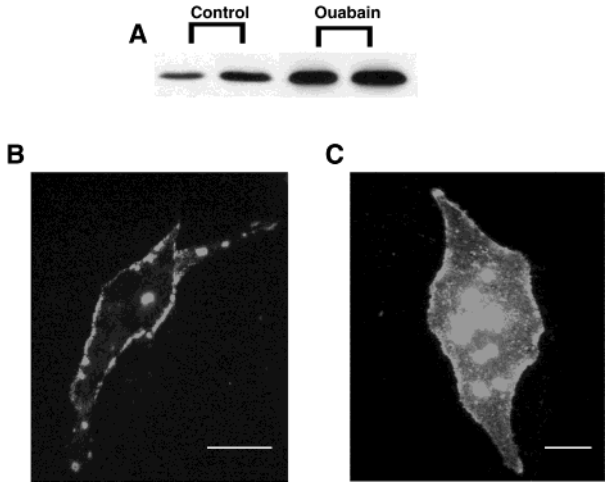

FIGURE 4: Effect of ouabain on FGF-2 secretion. (A) Lysates were prepared from CV-1 cells transiently transfected with FLAG-tagged FGF-2 and cultured in the presence or absence of 100 μM ouabain. Proteins (14 μg/lane) were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the anti-FLAG M2 mAb. (B and C) Localization of FLAG-tagged FGF-2 by immunofluorescence microscopy. FLAG-tagged FGF-2 was transiently expressed in CV-1 cells, and cells were cultured in the absence (B) or presence (C) of 100 mM ouabain. Cells were stained with a 1:2000 dilution of the anti-FLAG M2 mAB and visulaized with rhodamine red-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch). The bar represents 25 μm.

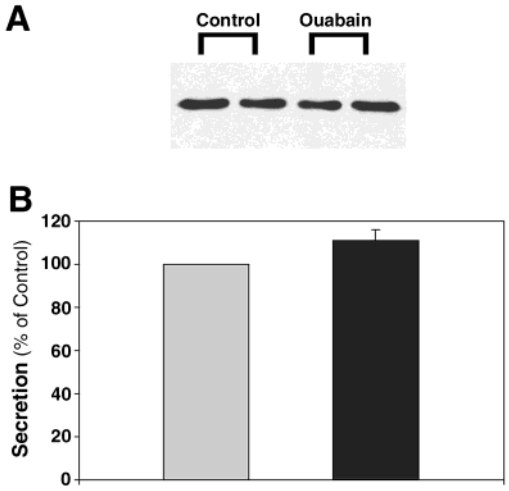

FIGURE 5: Rescue of FGF-2 secretion by the rat α1 subunit. CV-1 cells stably expressing the ouabain-resistant rat α1 subunit were transfected with FLAG-tagged FGF-2. Aliquots of medium were collected from cells grown in the presence or absence of 100 μM ouabain. Proteins were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed using the anti-FLAG M2 mAb. (A) Western blot. (B) Quantitation of bands by laser densitometry. The gray bar represents the data from cells grown in the absence of ouabain. The black bar represents the data obtained from cells grown in the presence of 100 μM ouabain. Bars represent the data obtained from two separate transfections each performed in duplicate. Error bars represent the SEM.

subunit to rescue ouabain-inhibitable FGF-2 secretion in primate cells. In this assay, the naturally ouabain-resistant rat α1 subunit was introduced into ouabain-sensitive CV-1 and HEK 293 cells, and stable transfectants were selected on the basis of resistance to 1 μM ouabain. The transfer of ouabain resistance indicates that the transfected α subunit can substitute for the endogenous α subunit of recipient cells (*26*). Because endogenous sodium pumps are poisoned in the presence of ouabain, rescue from ouabain cytotoxicity provides clear evidence that the transfected α subunit contributes to a functional Na,K-ATPase.

Using CV-1 cells stably expressing the ouabain-resistant rat α1 subunit, we assayed FGF-2 secretion in the presence or absence of 100 μM ouabain. The results are shown in Figure 5. No significant difference in FGF-2 export was observed between control and drug-treated cells. Expression of the rat α1 subunit in HEK 293 cells was also capable of supporting FGF-2 release in the presence of 100 μM ouabain (data not shown). These results constitute direct genetic evidence which shows that the Na,K-ATPase α1 subunit participates in the mechanism responsible for FGF-2 secretion.

Specificity of Na,K-ATPase Subunits for FGF-2 Secretion. The ability of the ouabain-resistant α1 subunit to rescue FGF-2 secretion in ouabain-sensitive cells provides a means for assessing whether other Na,K-ATPase α subunit isoforms are capable of supporting FGF-2 export. To determine whether the Na,K-ATPase α2 and α3 isoforms can also participate in FGF-2 release, ouabain-resistant versions of these isoforms (*23*, *24*) were used to generate stable α2- or α3-expressing ouabain-resistant CV-1 cell lines. Each of the cell lines was transiently transfected with FLAG-tagged FGF-2 and export detected in medium samples with the FLAG-specific mAb. CV-1 cells expressing either the α2

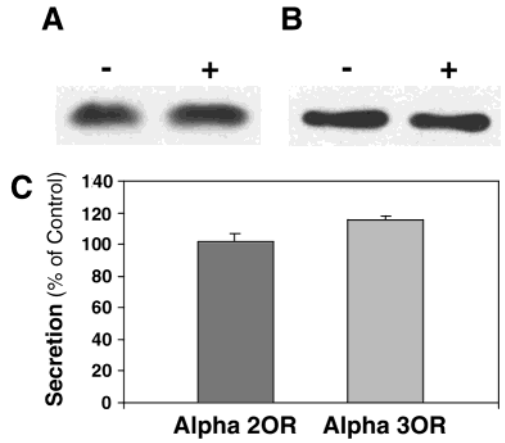

FIGURE 6: Rescue of FGF-2 secretion by ouabain-resistant α2 and α3 subunits. CV-1 cells stably expressing the mutated, ouabain-resistant (OR) forms of the rat α2 (A) and α3 (B) subunits were transfected with FLAG-tagged FGF-2. Aliquots of medium were collected from cells grown in the presence (+) or absence (−) of 100 μM ouabain. Proteins were fractionated by electrophoresis on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed using the anti-FLAG M2 mAb. (A and B) Western blot. (C) Quantitation of bands by laser densitometry. Bars represent the data obtained from one transfection performed in triplicate. Error bars represent the SEM.

(Figure 6A,C) or α3 (Figure 6B,C) subunits exhibited approximately equal levels of released FGF-2 in the presence or absence of 100 μM ouabain. These results indicate that both the α2 and α3 subunits have the capacity to support FGF-2 export from transfected CV-1 cells.

CV-1 cells expressing the rat α4 subunit were also tested for their capacity to release FGF-2. The α4 isoform is a testis-specific α subunit which is resistant to low ouabain concentrations (*22*). CV-1 cells expressing the α4 subunit exhibit an IC_{50} for ouabain of ~1.5 μM, compared to an

IC_{50} for ouabain of >100 μM for cells expressing the ouabain-resistant rat α1 subunit (*22*). Surprisingly, cells expressing the α4 subunit exhibited no significant reduction in the level of FGF-2 release during a 4 h treatment period with 100 μM ouabain (data not shown), a concentration of drug that should have inhibited the activity of α4-containing sodium pumps. To further investigate the mechanism of α4-mediated FGF-2 release, we compared the kinetics of FGF-2 export from CV-1 cells expressing either the rat α1 or rat α4 subunit. Cells were grown in the presence of 100 μM ouabain, and medium samples were assayed for the presence of FGF-2 at 30 min intervals. As shown in Figure 7A, the level of FGF-2 export increased in an approximately linear fashion ($R^2 = 0.90$) in cells expressing the ouabain-resistant rat α1 subunit. In cells expressing the rat α4 subunit, however, the level of FGF export increased during the first 60 min of ouabain treatment and then plateaued for the remainder of the experiment (Figure 7B). These results suggest that in the presence of 100 μM ouabain, it takes ~1 h to inhibit both α4 subunit activity and FGF-2 release. We also examined the effect of pretreating α4-expressing CV-1 cells with 100 μM ouabain for 2 h prior to initiating the secretion assay. Compared to that in untreated α4-expressing or ouabain-treated α1-expressing cells, pretreatment of α4-expressing cells with ouabain caused a >90% reduction in the level of FGF-2 export (Figure 7C−E). These results are consistent with the notion that the Na,K-ATPase α4 subunit can support FGF-2 export. Our data do not preclude the possibility that α4-mediated FGF-2 export is less sensitive to ouabain inhibition than is α4-mediated Na,K-ATPase activity.

Interaction between FGF-2 and the Na,K-ATPase. The Na,K-ATPase α subunit and FGF-2 have previously been reported to interact in transfected COS-1 cells (*10*). We utilized co-immunoprecipitation to determine whether there is an interaction between the Na,K-ATPase α1 subunit and FGF-2 transiently expressed in CV-1 cells. An anti-Na,K-ATPase α1 mAb was used to immunoprecipitate the endogenous α1 subunit from CV-1 cell lysates. Following electrophoretic separation and transfer to a filter, we probed the filter with an anti-FGF-2 polyclonal antibody. As depicted in Figure 8A, we detected an ~18 kDa FLAG-reactive band in lysates prepared from transfected, but not mock-transfected, CV-1 cells. To verify that the 18 kDa band represented FGF-2, we used an anti-FLAG polyclonal antibody to immunoprecipitate FLAG-tagged FGF-2 from cell lysates. Using the anti-FLAG M2 mAb (Figure 8B) or an anti-FGF-2 mAb (Figure 8C) as probes, we detected the 18 kDa band in cell lysates and the protein fraction immunoprecipitated from transiently transfected cells. These results provide strong evidence for physical interaction between the Na,K-ATPase α1 subunit and FGF-2 polypeptides. It is not known, however, whether the proteins interact directly or indirectly via an additional partner or partners.

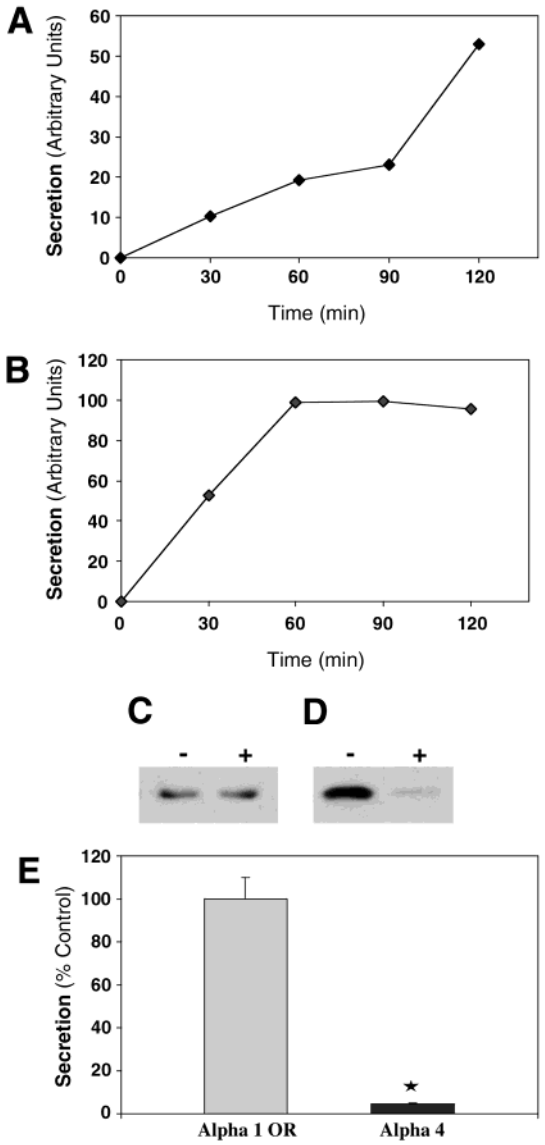

FIGURE 7: Effect of ouabain on α4 subunit-mediated FGF-2 release. (A and B) Time course for ouabain inhibition of FGF-2 export. CV-1 cells stably expressing the ouabain-resistant rat α1 (A) or rat α4 subunit (B) were transiently transfected with FLAG-tagged FGF-2. Aliquots of medium were collected from cells grown in the presence of 100 μM ouabain and assayed for FGF-2 release as described in Experimental Procedures. (C−E) Pretreatment of α4-expressing cells with ouabain blocks FGF-2 secretion. CV-1 cells expressing the ouabain-resistant rat α1 (C) or rat α4 (D) subunit were grown in the absence (−) or presence (+) of 100 μM ouabain for 2 h prior to initiating the secretion assay. (C and D) Western blot. (E) Quantitation of bands by laser densitometry. Bars represent the data obtained from one transfection performed in triplicate. Error bars represent the SEM. The asterisk indicates statistically significant reduction of the level of FGF-2 export in the presence of 100 μM ouabain ($p < 0.005$).

DISCUSSION

We have examined the relationship between Na,K-ATPase activity and secretion of the mitogen FGF-2 from transfected primate cells. Previous experiments demonstrated a link between Na,K-ATPase activity and the level of FGF-2 export based on the sensitivity of FGF-2 secretion to the Na,K-ATPase-specific inhibitor ouabain (*10*). To further elucidate the basis for FGF-2 secretion, we have utilized an assay system that relies on the ability of a stably transfected ouabain-resistant Na,K-ATPase α subunit to confer ouabain resistance upon ouabain-sensitive cells. Using this assay, we show that ouabain-mediated inhibition of FGF-2 export can be rescued via expression of each of several ouabain-resistant Na,K-ATPase α subunit isoforms. These results are in contrast with previous data showing that transient expression of the ouabain-resistant rat α1 subunit in COS-1 cells actually inhibited the export of FGF-2 (*10*). While the reason for this

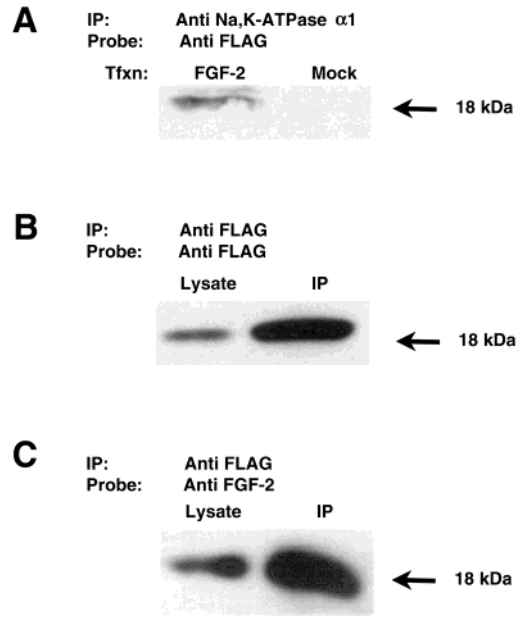

FIGURE 8: Interaction between the Na,K-ATPase and FGF-2. (A) Western blot of proteins immunoprecipitated from lysates of CV-1 cells mock-transfected or transfected with FLAG-tagged FGF-2. Lysates were immunoprecipitated using the anti Na,K-ATPase α1 mAb and probed using an anti-FLAG polyclonal antibody. (B and C) Western blots of cell lysates or proteins immunoprecipitated from CV-1 cells transiently transfected with FLAG-tagged FGF-2. Lysates were immunoprecipitated using an anti-FLAG polyclonal antibody. Proteins were fractionated on an SDS-containing 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with either the anti-FLAG M2 mAb (B) or an anti-FGF-2 mAb (C). The position of the 18 kDa form of FGF-2 is indicated by the arrows.

discrepancy is not entirely clear, it is possible that high expression levels of the transiently expressed α1 subunit could have saturated or otherwise inhibited the FGF-2 secretion pathway. The ability of ouabain-resistant forms of the α subunit to rescue FGF-2 export provides unequivocal evidence that the Na,K-ATPase participates in the mechanism mediating FGF-2 release.

Classic cell biological experiments have established that most secretory proteins contain a hydrophobic leader sequence that serves as a signal for secretion (*29*). Two FGF family members, FGF-1 and FGF-2, lack the hydrophobic signal sequence, yet are exported from cells (*11*). Several lines of evidence suggest that secretion of polypeptides lacking a signal sequence occurs through a novel release process distinct from that of the ER/Golgi secretory pathway (*10*, *30*, *31*). However, the components of this alternative export pathway have not yet been identified. In cultured cells, secretion of FGF-2 appears to occur constitutively (*16*), whereas FGF-1 release requires heat shock (*32*). Recent experiments carried out in NIH 3T3 cells have revealed that synaptotagmin-1, a protein normally associated with neuronal synaptic vesicles, is one of the components required for FGF-1 release through a non-ER/Golgi release pathway (*30*). It is not clear from these experiments whether FGF-1 release involves vesicular components of the classical secretory pathway, or is regulated via a nonvesicular exocytotic mechanism. It will clearly be of interest to determine whether Na,K-ATPase activity, which is necessary for FGF-2 secretion, is also a component of the FGF-1 export pathway. To address this issue, it will be necessary to analyze whether FGF-1 release can be blocked by ouabain in transfected primate cells, because NIH 3T3 cells endogenously express a ouabain-resistant Na,K-ATPase α1-containing enzyme (*33*). The ability to monitor FGF-1 secretion in CV-1 cells would then allow us to determine whether FGF-1 export and FGF-2 export occur via the same pathway or different exocytotic pathways.

The data presented in this report provide compelling evidence that supports the notion that Na,K-ATPase is at least one of the components required for FGF-2 release in CV-1 and HEK 293 cells. Consistent with this view, it has recently been shown that inhibition of Na,K-ATPase by oleandrin (a hot-water extract of the plant *Nerium oleander*) prevents FGF-2 export from human prostate PC3 and DU145 cells (*34*). In contrast, it has been reported that secretion of FGF-2 is sensitive to the drug probenecid in AIDS-associated Kaposi's sarcoma cells (*34*) and the osteosarcoma cell line MG-63 (*31*). Probenecid is a putative inhibitor of the multidrug resistance-associated protein (MRP) (*36*), an integral membrane protein that belongs to the superfamily of ATP-binding cassette transporters (*37*). We have carried out Western blot analysis using an anti-MRP mAb (QCRL-1; Centocor, Inc.) and have failed to detect expression of MRP protein in CV-1 cells (J. P. Dahl et al., unpublished results). Moreover, FGF-2 secretion in transfected CV-1 cells appears to be insensitive to treatment with 5 μM probenecid (J. P. Dahl et al., unpublished results), a concentration of drug that effectively blocks FGF-2 export in MG-63 and AIDS-associated Kaposi's sarcoma cells (*31*, *35*). While these experiments argue against the involvement of MRP in FGF-2 release in the CV-1 cell system, it is entirely possible that there are additional protein components besides Na,K-ATPase that participate in the mechanism leading to FGF-2 export.

The immunoprecipitation data presented here, as well as by others (*10*), imply an interaction between the Na,K-ATPase and FGF-2. This interaction may be direct, or the two proteins could interact indirectly as components of a multiprotein complex involved in the regulation of FGF-2 release. An initial yeast two-hybrid screen between FGF-2 and cytoplasmic domains of the Na,K-ATPase α and β subunits failed to uncover a direct interaction between FGF-2 and the ATPase (*38*). It is possible that the failure to detect interaction in the two-hybrid system could reflect the fact that the Na,K-ATPase−FGF-2 interaction is dependent on the ATPase assuming its correct, fully folded configuration. Alternatively, these results may imply that the interaction between Na,K-ATPase and FGF-2 is indirect, and involves the participation of one or more additional components. If this turns out to be the case, using FGF-2 as bait in a yeast two-hybrid screen may allow us to identify additional protein components that mediate the Na,K-ATPase−FGF-2 interaction.

To better understand the mechanism responsible for FGF-2 export, we utilized a somatic cell genetic strategy to examine whether distinct Na,K-ATPase α subunit isoforms had the capacity to support FGF-2 export from transfected cells. Together, our data point to the fact that the α1, α2, and α3 subunits can each rescue primate cells from ouabain cytotoxicity as well as ouabain-inhibitable FGF-2 export. The α4 subunit, a testis-specific α subunit isoform that confers low levels of ouabain resistance upon ouabain-sensitive cells

(*22*), also supported FGF-2 release. At first glance, α4-containing sodium pumps appeared to be capable of promoting FGF-2 export at concentrations of ouabain that should have inhibited α4-mediated Na,K-ATPase activity. Upon further inspection, we found that in α4-expressing cells, ~2 h of treatment with 100 μM ouabain led to a marked inhibition of FGF-2 export (Figure 7). These results imply that FGF-2 export may be less sensitive to ouabain inhibition than Na,K-ATPase activity. Alternatively, it is possible that FGF-2 export involves a population of Na,K-ATPases with restricted accessibility to drug. In either case, it seems reasonable to suggest that FGF-2 export and ion transport may represent functionally distinct properties of the α4 subunit (and possibly other α subunit isoforms).

In addition to FGF-1 and FGF-2, several other proteins lacking a signal sequence appear to be secreted from cells. For example, it has been reported that IL-1β is secreted from cells through the action of of P-glycoprotein (*39*), while CNTF is released by cultured astrocytes (*40*). It will be of much interest to determine whether Na,K-ATPase plays a role in the export of either IL-1 β or CNTF and, if so, whether a particular sodium pump isoenzyme shows specificity for IL-1 β or CNTF release. Understanding the role of the Na,K-ATPase in regulating secretion of these molecules may also shed light on the significance of Na,K-ATPase isoenzyme diversity.

REFERENCES

1. Cantley, L. C. (1981) *Curr. Top. Bioenerg. 11*, 201−237.
2. Sweadner, K. J. (1989) *Biochim. Biophys. Acta 988*, 185−220.
3. Jorgenson, P. L. (1974) *Biochim. Biophys. Acta 356*, 53−67.
4. Lingrel, J. B., and Kuntzweiler, T. A. (1994) *J. Biol. Chem. 269*, 19659−19662.
5. Levenson, R. (1994) *Rev. Physiol. Biochem. Pharmacol. 123*, 1−45.
6. Malik, N., Canfield, V., Beckers, M., Gros, P., and Levenson, R. (1996) *J. Biol. Chem. 271*, 22754−22758.
7. Lemas, M. V., Yu, H.-Y., Takeyasu, K., Kone, B., and Fambrough, D. (1994) *J. Biol. Chem. 269*, 18651−18655.
8. Schmalzing, G., Ruhl, K., and Gloor, S. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1136−1141.
9. Crambert, G., Hasler, U., Beggah, A. T., Yu, C., Maodyanov, N., Horisberger, J.-D., Lelievre, L., and Geering, K. (2000) *J. Biol. Chem. 275*, 1976−1986.
10. Florkiewicz, R. Z., Anchin, J., and Baird, A. (1998) *J. Biol. Chem. 273*, 544−551.
11. Mason, I. J. (1994) *Cell 78*, 547−552.
12. Sugi, Y., Sasse, J., and Lough, J. (1993) *Dev. Biol. 157*, 28−37.
13. Jung, J., Zheng, M., Goldfarb, M., and Zaret, K. S. (1999) *Science 284*, 1998−2003.
14. Folkman, J., and Shing, Y. (1992) *J. Biol. Chem. 267*, 10931−10934.
15. Nelson, R. M., and Long, G. L. (1989) *Anal. Biochem. 180*, 147−151.
16. Florkiewicz, R. Z., Majack, R. A., Buechler, R. D., and Florkiewicz, E. (1995) *J. Cell. Physiol. 162*, 388−399.
17. Kozak, M. (1978) *Cell 15*, 1109−1123.
18. Florkiewicz, R. Z., and Sommer, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3978−3981.
19. Prats, H., Kaghad, M., Prats, A. C., Klagsbrun, M., Lelias, J. M., Liauzun, P., Chalon, P., Tauber, J. P., Amalric, F., Smith, J. A., and Caput, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1836−1840.
20. Brewer, C. B., and Roth, M. G. (1991) *J. Cell Biol. 114*, 413−421.
21. Emanuel, J. R., Schulz, J., Zhou, X.-M., Kent, R. B., Housman, D., Cantley, L., and Levenson, R. (1988) *J. Biol. Chem. 263*, 7726−7733.
22. Underhill, A. D., Canfield, V. A., Dahl, J. P., Gros, P., and Levenson, R. (1999) *Biochemistry 38*, 14746−14751.
23. Canfield, V., Emanuel, J. R., Spickofsky, N., Levenson, R., and Margolskee, R. F. (1990) *Mol. Cell. Biol. 10*, 1367−1372.
24. Jewell, E. A., and Lingrel, J. B. (1991) *J. Biol. Chem. 266*, 29682−29687.
25. Fuller, S. A., Takahashi, M., and Hurrell, J. G. R. (1994) in *Current Protocols in Molecular Biology* (Ausubel, F. H., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.) Vol. 2, pp 11.5.1−11.5.3, John Wiley and Sons, New York.
26. Canfield, V. A., and Levenson, R. (1993) *Biochemistry 32*, 13782−13786.
27. McKeehan, W. L., Wang, F., and Kan, M. (1998) *Prog. Nucleic Acid Res. Mol. Biol. 59*, 135−176.
28. Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem. 261*, 11398−11403.
29. Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996) *Annu. Rev. Biochem. 65*, 271−303.
30. LaVallee, T. M., Tarantini, F., Gamble, S., Carreira, C. M., Jackson, A., and Maciag, T. (1998) *J. Biol. Chem. 273*, 22217−22223.
31. Aggarwal, S., and Gupta, S. (1998) *Int. J. Oncol. 13*, 1331−1334.
32. Jackson, A., Tarantini, F., Gamble, S., Friedman, S., and Maciag, T. (1995) *J. Biol. Chem. 270*, 33−36.
33. Fallows, D., Kent, R. B., Nelson, D. L., Emanuel, J. R., Levenson, R., and Housman, D. E. (1987*) Mol. Cell. Biol. 7*, 2985−2987.
34. Smith, J. A., McConkey, D. J., Lin, Y., Madden, T., Nutt, L. K., Cisneros, A., Felix, E., Vijeswarapu, M., Azadi, P., Ozel, H. Z., and Newman, R. A. (2000) *Proc. Am. Assoc. Cancer Res. 41*, 601−602 (abstract).
35. Gupta, S., Aggarwal, S., and Nakamura, S. (1998) *J. Clin. Immunol. 18*, 256−263.
36. Zaman, G. J. R., Versantvoort, C. H. M., Smit, J. J. M., Eijdems, E. W. H. M., Haas, M., Smith, A. J., Broxterman, H. J., Mulder, N. H., DeVries, E. G. E., Bass, F., and Borst, P. (1993) *Cancer Res. 53*, 1747−1750.
37. Cole, S. P., Bharadwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, A. U., Duncan, A. M., and Deeley, R. G. (1992) *Science 258*, 1650−1654.
38. Oh, J., and Lee, K. (1998) *Arch. Pharm. Res. 21*, 707−711.
39. Young, P. R., and Krasney, P. A. (1992) *Prog. Biomed. Res. 2*, 21−27.
40. Kamiguchi, H., Yoshida, K., Sagoh, M., Sasaki, H., Inaba, M., Wakamoto, H., Otani, M., and Toya, S. (1995) *Neurochem. Res. 20*, 1187−1193.

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